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EFFECTS OF INHALATION ANESTHETICS ON DECOMPOSITION OF KETONE BODIES IN RAT TISSUE SLICES*

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(Received for publication, January 7, 1959)

In a previous pepar effects of two kinds of inhalation anesthetics, diethyl ether (EE) and trichloroethylene (TCE), on the production of ketone bodies in rat liver slices were studied with the results that the production of ketone bodies in liver slices promoted by epinephrine administration, while it was reduced by EE anesthesis and CCl_4 administration, and was not influenced by TCE anesthesia (*1*).

As already known, the ketone bodies are produced mainly in liver and are decomposed in the extrahepatic tissues such as muscle or kidney. In this paper the effects of these inhalation anesthesia on the decomposition of ketone bodies and the comparison with the effect of epinephrine administration are reported.

EXPERIMENTAL

Detailed experimental procedures were almost the same as previously described (*1*). Kidney and diaphragm were taken out of the rats anesthetized with EE or TCE, and epinephrinized as well. The kidney was cut with free hand into several slices and the diaphragm was cut, exclusive of the middle zone, *pars membranacea*, into several pieces. These pieces of tissues were distributed into each Warburg flask as described in Table I.

The spontaneous decomposition of acetoacetate during shaking in Warburg flasks, was measured in the flask F without tissue slices and the correction was made for the measurements in the flasks A, C and E respectively. Acetoacetate was prepared by the method of Ljunggen (*2*). Acetoacetate and β-hydroxybutyrate were determined quantitatively by the method of Koide *et al.* (*3*).

In this experiment both Q_{O_2} and the amount of acetoacetate consumed and β-hydroxybutyrate produced were determined in duplicate and averaged.

Total and free carbohydrate content in diaphragm was determined by the modified method of Yamamoto (*1*).

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* This study was made possible through a grant in Aid for Therapeutic Research given from the Minister of Health and Welfare, and was reported at the 27th annual meeting of the Japanese Biochemical Society, Kyoto, April 1955.

TABLE I

Experimental Conditions

Main compartment	A	B	C[1]	D	E	F
Krebs-Ringer phosphate buffer	2.0	2.5	2.0	2.5	2.0	2.0 *ml.*
Tissue slices (dry weight)	Kidney	Kidney (10–20 *mg.*)	Kidney	Diaphragm (5–10 *mg.*)		—
Side arm						
Sod. acetoacetate[2]	0.5	—	0.5	—	0.5	0.5 *ml.*
Center well						
10% KOH[3]	0.2	0.2	0.2	0.2	0.2	0.2 *ml.*

Flasks were incubated in an atmosphere of 100 per cent O_2 in Warburg water bath at 37.5°, with constant shaking at the rate of 110–120 strokes per minute for 60 minutes.

1) Gas phase: 100 per cent N_2
2) Contains 1.56–1.04 *M* in 0.5 ml.
3) Rolls of filter-paper were inserted.

RESULTS AND DISCUSSION

I. Oxygen Uptake and Acetoacetate Decomposition in Diaphragm and Kidney—The oxygen uptake in diaphragm and kidney slices, with or without acetoacetate, was measured and the difference of this oxygen uptake was compared with the amount of acetoacetate consumed. (Tables II, and III)

TABLE II

Oxygen Uptake and Acetoacetate Consumption in Rat Diaphragm

Each value represents the average of 7 experiments.

Group	Oxygen Uptake		Acetoacetate[1] consumed
	With acetoacetate	Without acetoacetate	
	$(-Q_{O_2})$	$(-Q_{O_2})$	(μM)
Untreated control	5.2 ± 1.1	4.0 ± 1.2	1.6 ± 0.8
Epinephrinized	5.7 ± 0.8	4.5 ± 0.8	1.5 ± 0.6
Anesthetized with TCE	5.0 ± 1.2	4.1 ± 1.1	1.6 ± 0.7
Anesthetized with ethyl ether	4.4 ± 0.9	3.2 ± 0.5	1.9 ± 0.9

1) Represents values expressed in *μM* of acetoacetate consumed per 100 mg. of dry weight diaphragm per 60 minutes.

These results show that oxygen uptake and acetoacetate consumption of the three groups treated respectively with epinephrine, TCE and EE did not differ significantly from the control group in diaphragm, but they were a little lower in kidney. In the latter the ratio of acetoacetate consumed to oxygen uptake was almost the same as in the control and from this fact it may be considered that there are no qualitatively different aerobic metabolism of acetoacetate among those groups.

TABLE III

Oxygen Uptake and Acetoacetate Consumption in Rat Kidney Slices

Each value represents the average of 7 experiments.

Group	Oxygen Uptake: With acetoacetate (A)	Oxygen Uptake: Without acetoacetate (B)	Acetoacetate consumed (C)	Ratio[1] $C/A-B \times 22.4$
	($-Q_{O_2}$)	($-Q_{O_2}$)	(μM)	
Untreated control	21.8±2.7	18.3±2.7	6.0±1.2	0.38
Epinephrinized	23.2±2.3	20.6±2.0	4.5±0.8[2]	0.39
Anesth. with TCE	20.3±1.8	18.0±2.0	3.8±0.7[2]	0.37
Anesth. with ethyl ether	21.7±2.5	18.7±2.3	4.6±1.0[2]	0.35

1) Represents the molar ratios of acetoacetate consumed to extra oxygen uptake.

2) Significantly different from the mean value for the control ($P<0.05$).

II. Variation of Carbohydrate Content in Diaphragm—It has been generally accepted that the metabolism of acetoacetate in tissues are influenced by their nutritional state. For example it was found that liver slices of normally nourished rats produce acetoacetate at low rate, and that nearly all of the ketone bodies thus produced can be oxidized completely.

However on fasted rats acetoacetate production increased and its oxidation decreased (*4*). In order to study this point in case of diaphragm, variations of free and total carbohydrate content among those groups were determined and the results are represented in Table IV.

TABLE IV

Total and Free Carbohydrate in Rat Diaphragm

Each value represents the average of 7 experiments.

Group	Total carbohydrate	Free sugar
	(%)	(%)
Untreated control	2.80±0.39	0.88±0.23
Epinephrinized	1.89±0.14[1]	0.38±0.20[1]
Anesthetized with TCE	2.17±0.52[1]	0.64±0.21
Anesthetized with ethyl ether	2.38±0.42	0.50±0.16[1]

1) Significantly different from the mean value for the controls ($P<0.05$)

From this table it can be seen that while both free and total carbohydrate decreased significantly in the epinephrinized group, either free or total carbohydrate alone decreased significantly in the case of EE or TCE anesthesia respectively. The diminution in the content of total carbohydrate or free sugar in diaphragm, may indicate the decreased nutritional state, which bears some relation to the decreased decomposition of acetoacetate in this tissue.

III. Anaerobic Metabolism of Ketone Bodies in Kidney Slices—The amount of acetoacetate consumed and β-hydroybutyrate produced in kidney slices were determined in nitrogen atmosphere. Results are shown in Table V.

TABLE V

Acetoacetate Consumption and β-Hydroxybutyrate Production in Rat Kidney Slices in Nitrogen Atmosphere

Each value represents the average of 6 experiments.

Group	Acetoacetate¹⁾ consumed (*D*)	β-hydroxybutyrate¹⁾ produced (E)	Ratio (E/D)
	(μM)	(μM)	
Untreated control	3.0±1.0	0.7±0.2	0.23
Epinephrinized	2.8±0.6	1.3±0.3²⁾	0.46
Anesth. with TCE	2.8±0.9	1.2±0.3²⁾	0.43
Anesth. with ethyl ether	3.4±0.6	1.3±0.3²⁾	0.38

1) Represent values expressed in μM of acetoacetate consumed or β-hydroxybutyrate produced in N_2 per 100 mg. of dry weight kidney per 60 minutes.

2) Significantly different from the mean value for the control ($P<0.05$).

From this table, it is evident that the consumption of acetoacetate showed no reduction under anaerobic conditions, but the ratio of β-hydroxybutyrate produced to acetoacetate consumed showed significant increase in every group compared with the control.

These results may be explained as follows. Under anaerobic condition, acetoacetyl-CoA*, formed by the activation of acetoacetate, shows the tendency to accept hydrogen by the aid of ketoreductase from reduced form of DPN which exists predominantly under such condition. β-Hydroxybutyryl CoA thus formed abundantly, liberates free β-hydroxybutyrate into the media. On the other hand, the breakdown of β-hydroxybutyrate is inhibited by two ways:

1) By the decrease of activation.

The activation of β-hydroxybutyrate may be performed as follows:

$$\beta\text{-hydroxybutyrate}+\text{CoA}+\text{ATP}\longrightarrow\beta\text{-hydroxybutyryl}-\text{CoA}+\text{AMP}+\text{PP}$$

The oxidative resynthesis of ATP from AMP thus formed, is decreased under anaerobic condition and by the action of narcotics, which are known to inhibit the oxidative synthesis of ATP (*5*). These decreased resynthesis of ATP leads to the decrease of the activation of β-hydroxybutyrate.

2) By the inhibition of oxidation of DPNH.

The reduction of acetoacetyl-CoA, formed by the activation of acetoacetate, is performed by DPNH which exists predominantly under anaerobic condition. However the oxidation of DPNH by mitochondria to produce

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* Following abbreviations are used. CoA: Coenzyme A; ATP: adenosine triphosphate; PP: pyrophosphate; AMP: adenosine monophosphate; DPN: and DPNH diphosphopyridine nucleotide and its reduced form.

DPN, is inhibited by narcotics such as EE or barbiturates (*6*). Consequently the oxidation of β-hydroxybutyryl-CoA is suppressed resulting in the decrease of acetoacetate production by the reverse process.

Both the increased production and the decreased decomposition of β-hydroxybutyrate lead to the accumulation of this substance in the media.

SUMMARY

Rats were anesthetized with ethylether or trichloroethylene to study the effects of those narcotics on the decomposition of acetoacetate in kidney slices or diaphragm under aerobic or anaerobic conditions, on the anaerobic production of β-hydroxybutyrate in kidney slices, and on the content of carbohydrate in diaphragm. The results were compared with those of control or epinephrinized rats and summarized as follows:

1. While the consumption of acetoacetate in kidney is lowered by ethylether or trichloroethylene anesthesia as well as by epinephrine administration. that in diaphragm is not influenced by these treatments.
2. Anaerobic production of β-hydroxybutyrate in kidney is increased by these treatments.
3. As regards the content of carbohydrate in the diaphragm, both free sugars and total carbohydrate decreased significantly in the epinephrinized group, whereas in the case of ethylether or trichloroethylene anesthesia the decrease of either free sugar or total carbohydrate alone was significant.

Thanks are due to Prof. Emeritus Y. Sueyoshi of the Keio University for invaluable advice and encouragement.

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THE ION-EXCHANGE CHROMATOGRAPHY OF THE COPROPORPHYRINOGEN-SYNTHESIZING ENZYME CONTAINED IN CHICKEN ERYTHROCYTES

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The Institute of Public Health, Tokyo)

(Received for publication, July 17, 1959)

There have been many works to clarify the mechanism of porphyrin and heme biosynthesis.

For the past ten years, majority of chemical path way for heme biosynthesis has already been established by Shemin, Rittenberg, Granick, Altman, Salomon, and others in the United States and by Rimington, Neuberger, Dresel, Falk, and others in the United Kingdom (*1*, *2*).

The remaining problem now is the mechanism of formation of the porphyrin ring from PBG (porphobilinogen) and also of its further decarboxylation.

Recently, Granick (*3*) and Bogorad (*4*) reported the separation of a few enzymes related to above reactions and they isolated and purified three enzymes taking part in the reaction forming coproporphyrinogens from ALA (δ-aminolevulinic acid), and also an enzyme taking part in a reaction creating a difference between the I and III-types of porphyrin ring.

The purpose of this work is to separated the coproporphyrinogen-synthesizing enzyme from hemoglobin in the supernatant of the hemolysate of the chicken erythrocytes, and further to purify it.

For this purpose an ion-exchange chromatography was employed in this work. The ion-exchange resin used in the procedure was Amberlite IRC-50 which was considered to be most adequate for the separation of enzyme protein and hemoglobin (*5*, *6*).

EXPERIMENTAL AND RESULTS

About 500 ml. of citrated chicken blood (soon after bleeding) was centrifuged and erythrocytes were washed five times with 1.1 per cent KCl. The washed erythrocytes were then hemolysed with 2 volumes of water (*6*) at 4° for 2 hours and homogenized in a Waring blendor for 3 minutes at a low temperature. The homogenate was centrifuged at $10^5 \times g$ for 60 minutes and the supernatant was used as the crude enzyme solution.

Two milliliter of this solution was chromatographed through a column (2.8×40 cm) packed with Amberlite IRC-50 (100–300 mesh). The chromatography was carried out at pH 6.3. Solutions of very low ionic strength, such as, redistilled water, $10^{-4}\,M$, $3\times10^{-4}\,M$, or $2\times10^{-3}\,M$ phosphate buffer, were used as the developing solvent.

The results of chromatography are shown in Fig. 1. Contrary to the result of the previous experiment (*5*) in which 0.1 *M* phosphate buffer was used and only one band moved after separating from the hemoglobin band, a more complicated mode of separation was observed.

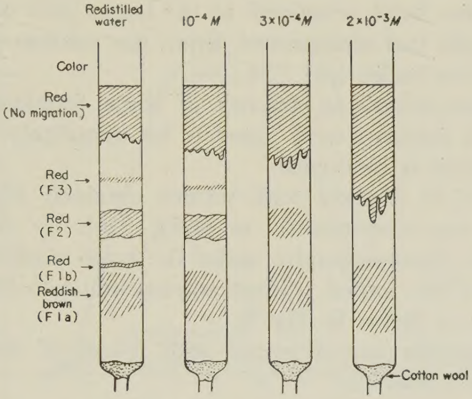

FIG. 1. Mode of separation of the supernatant of chicken erythrocytes homogenate in ion-exchange chromatography.

Ion-exchange resin: Amberlite IRC-50 (100-300 mesh). pH: 6.3. Developing solution: Phosphate buffer (pH 6.3) of concentrations specified in the figure.

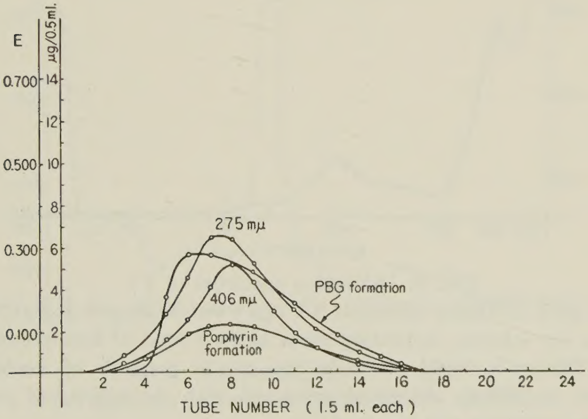

FIG. 2. Rechromatography of F 1.

The conditions of chromatography were the same as for Fig. 1. The developing solution used was $10^{-4}\,M$ phosphate buffer. The absorption at 275 mμ is related to the amount of protein. The absorption at 414 mμ is related to the amount of hemoprotein. PBG formation from ALA is dependent on the amount of ALA dehydrase. Porphyrin formation from PBG is related to the amount of other porphyrinogen synthesizing enzyme.

Furthermore, it was noted that the separation seemed to change even with a small change in ionic concentration of the solvent used. The usual mode of separation was considered to be that obtained at an ionic concentration of $10^{-4}\,M$, as shown in Fig. 1. The separated three bands were each collected in a fraction collector and were designated as F1, F2, and F3.

The hemoglobin band remained at the upper part of the resin column after the other bands had disappeared from the column which was eluted with $0.5\,M$ phosphate buffer (pH 7.5).

The porphyrin-synthesizing activity of these fractions was investigated. F3 and hemoglobin fractions were found to be completely inactive as for as porphyrin biosynthesis is concerned.

About 30 ml. of F1 fraction (total volume obtained from 2 ml. of crude enzyme solution) was concentrated to about 2 ml. by freezing and it was again submitted to chromatography under the same condition to the first one.

The fraction of the second column corresponding to F1 was collected by a fraction collector as shown in Fig. 2.

Absorption spectrum was measured with 0.4 ml of the 1.5 ml. fractions in each tube (Figs. 2 and 3).

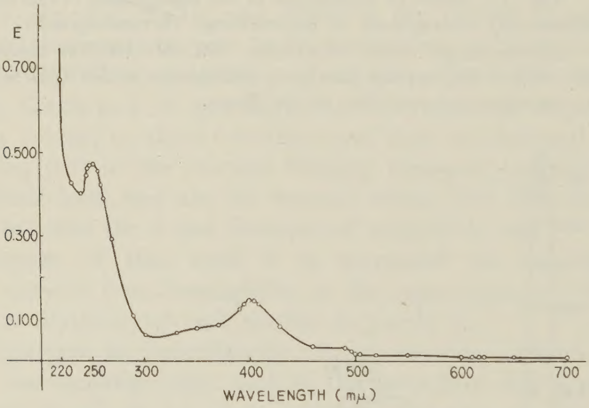

FIG. 3. Absorption spectrum of F 1.

The peak at 406 mμ shifted to 420 mμ when KCN and $K_3F_e(CN)_6$ were added to the solution, indicating them to be a kind of hemoprotein. The peak at 250 mμ is considered to be due to the presence of nucleic acid. The peak at 406 mμ decreased compared with the activity of protein as the chromatography was repeated.

To each of another two 0.4 ml. portions, 6 μg. of ALA* and 4 μg. of PBG** dissolved in 0.2 ml. of $0.05\,M$ phosphate buffer (pH 7.0) were respectively added, and both solutions were incubated aerobically at 37° for 3 hours.

After stopping the reaction by adding 3.5 ml. of $1\,N$ HCl and one drop of 0.05 per cent iodine solution to each incubation mixture, 3.0 ml. of the supernatant was submitted to the measurement of Soret band absorption and then of color reaction by Ehrlich reagent.* The former was used for the calculation of total porphyrin formed and the latter for the PBG formed.**

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* ALA=δ-aminolevulinic acid, supplied by the Daiichi chemical Co., Ltd. (Japan).

** PBG=porphobilinogen, synthesized from ALA by Gibson's procedure (7).

The results shown in Fig. 2 indicate a certain grade of separation between ALA-dehydrase and other porphyrinogen-synthesizing enzymes.

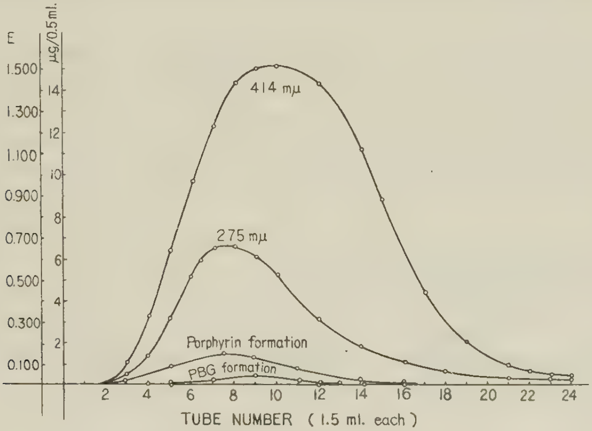

FIG. 4. Rechromatograhy of F 2
The conditions are all the same to those of Fig. 2.

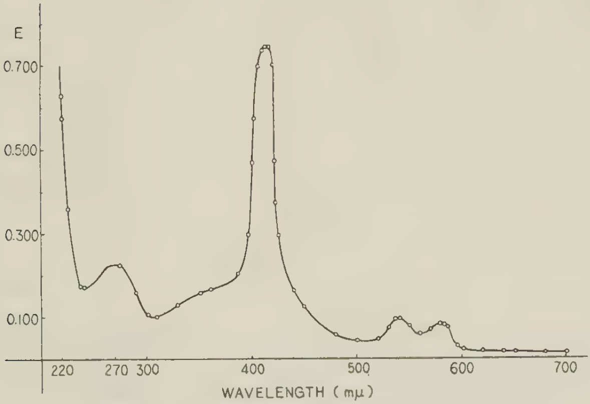

FIG. 5. Absorption spectrum of F 2.
This spectrum indicates a typical O_2-Hb absorption.

The results obtained with F 2 under the same experimental condition are summarized in Figs. 4 and 5.

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* Granick's procedure (*8*).

** These values are not considered to indicate the exact quantity of the substances formed, because the protein precipitate might have absorbed some amount of porphyrin and PBG which would be excluded from the calculation. However, they were neglected in this work.

As shown in Fig. 4, F 2 contains a rather small amount of porphyrin-synthesizing enzyme and a large amount of hemoglobin compared to F 1. The amount af ALA-dehydrase in F 2 was almost negligible.

To find whether such a difference between F 1 and F 2 is solely quantitative or not, further examination was carried out on their porphyrin-synthesizing activity.

A paper chromatographic method was employed to separate and also to detect the porphyrins formed in the incubation mixture. All F 1 and F 2 were lyophilized as described above in order to obtain a much stronger activity of porphyrin biosynthesis, because a stronger activity was considered to shorten the duration of incubation and eliminate the chance of various side reactions.

To 0.5 ml. of concentrated F 1 and F 2, 10 μg. of PBG was added as described above. This was incubated at 37° for 40 minutes. After stopping the reaction by adding 0.2 ml. of 5 N HCl and one drop of 0.05 per cent iodine solution an aliquots of the supernatant was immediately spotted at the original point on a sheet of filter paper. The paper was developed in a chamber in a NH_3 atmosphere with a solvent mixture of 2,4-lutidine* and water (1 : 1) at 5° for 30 hours in a dark room. The position of porphyrin

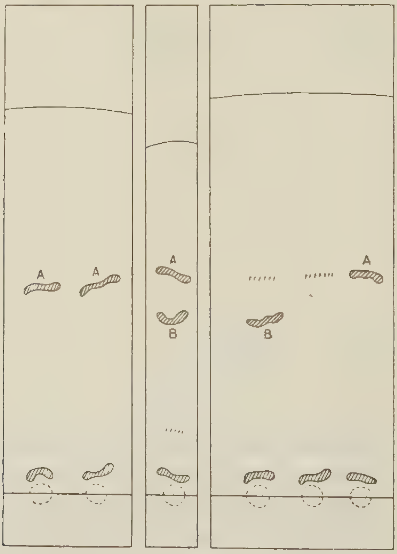

FIG. 6. Paper chromatogram of porphyrins formed by various enzyme solutions from PBG.

1.} Crude enzyme.
2.}
3. F 1.
4. F 1.
5. F 2.
6. Mixture of F 1 and F 2.

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* Recrystallized from the urine of congenital porphyria by Watson's (*11*) and Nicholas' methods (*12*).

spot on the paper chromatogram was detected under a mercury lamp.

In Fig. 6 is shown the paper chromatogram of the crude enzyme solution, F 1, F 2 and F 1+F 2. The majority of prophyrin spots on the paper chromatogram seemed to be those of uroporphyrin and coprophyrin.

The remarkable fact evidenced by Fig. 6 is that at least two kinds of coproporphyrin appeared on the chromatogram, one moving to the top and the other with less movement which were respectively designated as spots A and B. The nature of the difference between the two porphyrins will be discussed later.

Spot A is formed from the crude enzyme described above. It is also formed by F 1 or F 2 although the amount formed is usually very small in this case. Sport B is formed only from F 1.

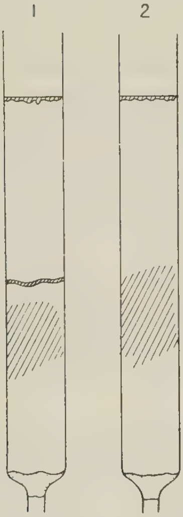

FIG. 7. Pattern of separation and migration in the ion-exchange chromatography.

1. Mixture of F 1 and dialysed F 2 after added with the concentrated outer solution of dialysed F 2.
2. Mixture of F 1 and dialysed F 2.

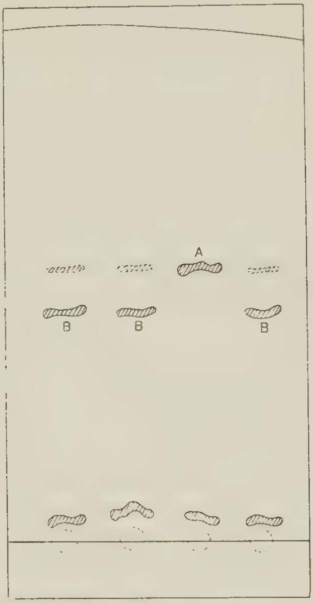

FIG. 8. Paper chromatogram of the porphyrins formed by various enzyme solutions from PBG.

1. F 1 only
2. Mixture of F 1 and solution (1)
3. Mixture of F 1 and solution (2)
4. Mixture of F 1 and solution (3)

Solution (1), (2) and (3) are respective eluates with water from the spot No. 1 (near the original point), spot 2 (R_f=P.1/0.3), and spot 3 (R_f=0.3–0.5) on the paper chromatogram after the concentrated outer solution of dialysed F 2 was developed by a mixture (4 : 1 : 1) of butanol, glacial acetic acid, and water.

Spot A completely corresponded to the standard coproporphyrin III* as far as the position of the spot (=R_f value) in the lutidine method was concerned.

Spot B did not have a difinite position but moved within a certain range near that of standard coproporphyrin I.

A rather interesting fact is that a relatively large amount of spot A alone was formed when F 2 was added to F 1. A similar additive effect was observed also by a boiled juice of F 2 or even by the concentrated outer solution of F 2 dialysed against redistilled water.

Therefore, F 2 was considered to contain some kind of effective substance which is dialysable and heat stable and related to the synthetic reaction for coproporphyrinogen.

This assumption was also confirmed by the following fact: When a mixture of F 1 and sufficiently dialysed F 2 was subjected to the column chromatography as described above, only one band was observed in the developed column, but, as shown in Fig. 7, when the concentrated outer solution of the dialysed F 2 was added to this mixture, the developed column was similar to that of the crude enzyme solution shown in Fig. 1. None of the other substances* tested showed such an effect.

Further examinations were made to clarify chemical structure of the substance.

The concentrated outer solution of dialysed F 2 was submitted to paper chromatography and developed with two kinds of solvent systems, one being a mixture (4 : 1 : 1) of butanol, glacial acetic acid, and water which was used for the separation of amino acids, peptides, *etc.*, and the other a mixture (5 : 15 : 0.5 : 10) of isoamyl alcohol, isopropyl alcohol, 75 per cent lactic acid, and 5 per cent trichloroacetic acid, which was used for the separation of various nucleotides.

In the resultant paper chromatogram, there was no spot with absorption at 260 mμ when developed with the latter system.

In the chromatogram developed with the former solvent system, three spots positive to ninhydrin were found, near original point (spot No. 1), at R_f 0.1–0.3 (spot No. 2), at R_f 0.3–0.5 (spot No. 3).

Of the aqueous extract of these spots, that of spot No. 2 was only effective for the formation of spot A described above when added to the incubation mixture of F 1 and PBG (Fig. 8).

Thus, the substance of spot 2 was found to be the true effective one.

The substance was extracted from the spot on the paper with water, hydrolysed with a dilute acid solution. The resulting hydrolysate was then submitted to paper chromatography as described above. Several ninhydrin-positive spots were obtained.

From these experimental data, it is assumed that this effective substance is a kind of polypeptide.

Further investigation about this substance is being continued.

DISCUSSION

The facts shown in Fig. 1 and Fig. 7 seem to suggest a very delicate interrelationship among the ionic states of hemoglobin molecule and other various enzyme protein molecules: For instance, each of the prophyrinogen synthesizing enzyme fractions in the ion-exchange chromatogram always included some amount of hemoprotein. Furthermore, the mode of their migration and separation changed very sensitively with a small change in ionic strength of the developing solution or with a simultaneous presence of another ionic substance, such as, some kind of polypeptides.

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* A few inorganic salts, amino acids, and gluthathione.

Upon what kind of difference among the chemical structures of their molecules the reason of these facts depends is a very interesting problem. However, further study of this problem was not carried out in the present work, because the chemical nature of the porphyrin-synthesizing enzymes and related substances is still under investigation and not yet fully clarified to discuss about their state of ionic charge either quantitatively or qualitatively.

The position of spot B shown in Fig. 6 was very close to that of standard coproporphyrin I as far as the R_f value in lutidine method was concerned. Therefore, if the porphyrin of spot B were coproporphyrin I, the effective substance described above should be related to isomerization of the porphyrin ring, but the result of paper chromatography* developed with dioxane to determine whether the porphyrin was coproporphyrin-I or not gave no conclusive evidence (details omitted).

Recently, according to Granick and Bogorad, two kinds of enzymes, the PBG desaminase and the isomerase, have been extracted and purified from various materials. The PBG-desaminase undergoes condensation with four molecules of PBG to form uroporphyrinogen-I. The isomerase acts on the intermediate formed in the reaction mixture of PBG-desaminase and PBG to form uroporphyrinogen-III by utilizing still PBG-desaminase activity and another PBG.

The dialysable and heat stable nature of the effective substance found in the present work does not agree with the facts found by Granick (*3*) and Bogorad (*4*). Therefore, it would be more appropriate to consider that the difference between the substances giving spot A and spot B originates from a difference in the number of carboxyl radicals.

Thus, the substance is considered to be an unknown cofactor which is related to the decarboxylation of certain porphyrinogen. Since F 1 can synthesize coproporphyrinogen giving spot B from PBG, the co-factor seems to be related only to the reaction of the substances spot B → spot A. In other words, it might take part in decarboxylation of only one or two carboxyl groups in the porphyrinogen whose chemical structure is an intermediate type between uroporphyrinogen and coproporphyrinogen. These mechanisms should be clarified in the near future.

Hoar and Heath (*14*) reported recently that a substance related to formation of coproporphyrinogen is present in *Rhodopseudomonas spheroides*. However, chemical properties and biochemical action of this substance were not reported in detail.

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* Benson's method (*13*).

SUMMARY

An attempt was made to separate the coproporphyrinogen-synthesizing enzyme and hemoglobin contained in chicken erythrocytes by ion-exchange chromatography, using Amberlite IRC-50.

Two fractions which have porphyrin-synthesizing activity were obtained from the $10^5\times-$ g-centrifuged supernatant of chicken erythrocyte homogenate when a developing solution of a very low ionic strength was used. Each fraction of the two found to involve the whole enzyme system which catalyzes the reaction of ALA→PBG→uroporphyrinogen→coproporphyrinogen.

Further clear separation of each enzyme from this enzyme system was unsuccessful, but one of the two fractions was found to contain an unknown substance which is considered to be a co-factor in the decarboxylation of two kinds of coproporphyrinogen.

The substance was dialysable and heat stable, and was suggested to be a polypeptide.

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The Journal of Biochemistry, Vol. 47, No. 2, 1960

STUDIES ON ENZYMATIC SYNTHESIS OF COCARBOXYLASE IN ANIMAL TISSUE

II. EXTERNAL FACTORS WHICH INFLUENCE THIAMINOKINASE ACTIVITY*

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(Received for publication, July 20, 1959)

It has been noted generally that the amount of TDP produced in the thiaminokinase reaction is extremely low at any time and the fact makes it difficult to elucidate further the detailed mechanism of this reaction. A speculative explanation of this phenomenon in yeast was proposed tentatively by Weil-Malherbe (*1*), in which some interrelationship was suggested to exist between the maximal yield of TDP and the content of apocarboxylase. The present paper deals with the effect of external factors which influence the TDP formation and cause cessation of the reaction.

METHODS AND MATERIALS

Enzyme preparation from rat liver and assay method used in this experiment were the same as those described in the previous paper (*2*) except that the enzyme preparation in a few experiments was highly purified after the description of the coming communication III of this series (*3*). Pyruvic oxidase was prepared from a dog heart according to the method of Jagannathan and Schweet (*4*). ATP was determined as described in a previous report (*5*) using myosin-B. Creatine phosphate, acetyl phosphate, and carbamyl phosphate were prepared by the methods of Ennor and Stocken (*6*), Bentley (*7*), and Jones *et al.* (*8*), respectively. Other materials used were the same as indicated before (*2*).

RESULTS AND DISCUSSION

(*A*) *Accelerating Effect of PEP on the Thiaminokinase Reaction*

It has been reported in the previous paper (*2*) that neither PEP nor A5P is effective as a phosphate donor by itself but, when used together, they become effective possibly by the action of pyruvate kinase contained in the enzyme preparation. According to the formula, 2PEP+A5P→ATP+2 pyruvate, the yield of TDP in this system cannot exceed one-half the amount of that when ATP is used as a phosphate donor, if PEP and ATP used are in the same molar concentration. However, the activity of the system of A5P+PEP was unexpectedly found to attain up to several times that of ATP when A5P was employed at a relatively low concentration. This fact suggests that PEP would act as a regenerator of ATP in the reaction so as to overcome the degradation of ATP and the inhibitory action of A5P which was reported in the previous paper (*2*). The production of ATP from A5P in the presence of PEP in this enzyme preparation was also described in that article (*2*).

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* The following abbreviations are used throughout this paper: ATP, ADP, and A5P, adenosine tri-, di-, and monophosphate-5′; PEP, phospho(enol)pyruvate; TDP, thiamine pyrophosphate (cocarboxylase); Tris, tris (hydroxymethyl) aminomethane.

Fig. 1 shows the individual effect of PEP and A5P on the reaction of TDP formation during one hour. The effect of A5P with constant concentration of PEP reached a maximal plateau at its relatively low concentration, and at higher concentrations A5P inhibited the reaction. This fact suggests that A5P promotes the reaction catalytically *via* ATP and shows its inhibition against ATP predominantly in higher concentrations. The catalytic role of A5P is supported by the fact that the reaction order against A5P at 3.3×10^{-4} to 2×10^{-3} *M* was of zero order when the velocity which reached constant was regarded as the initial velocity (Fig. 2). On the contrary, the promoting effect of PEP increased with considerable proportionality to its

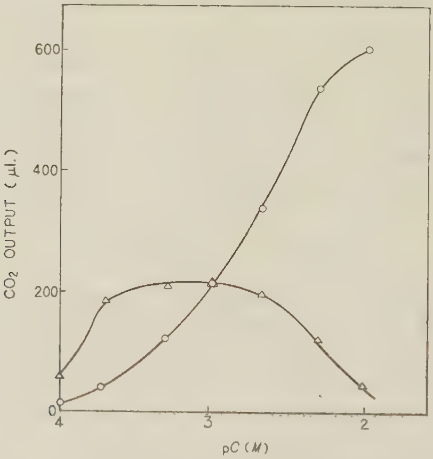

FIG. 1. Individual effect of variant PEP and A5P. —△— Activity of variant A5P when PEP was fixed at 10^{-3} *M*, —○— Activity of variant PEP when A5P was fixed at 10^{-3} *M*, p*C*(*M*), -log value of molar concentration of specified substrate.

Reaction conditions: 10^{-4} *M* thiamine; 3.3×10^{-3} *M* $MgSO_4$; PEP and A5P as specified; 2.3×10^{-2} *M* phosphate buffer of pH 7.4. Total volume, 3.0 ml. Incubation, 37° for 60 minutes. The determination of the TDP formed was carried out in the same way as described in the previous paper (*2*), and the corrected CO_2 output during 30 minutes was taken to indicate the amount of TDP.

The conditions are the same for all experiments shown in the following figures, unless otherwise stated.

concentration (Fig. 1) and it may be assumed that the amount of TDP produced is dependent upon the size of a PEP pool.

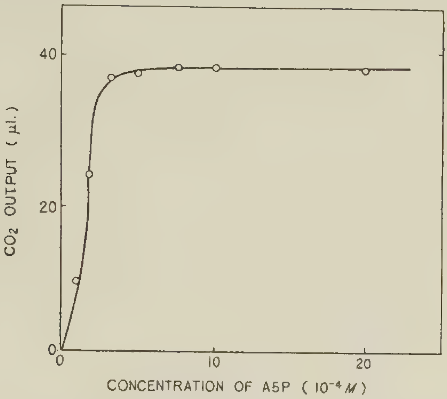

FIG. 2. Catalytic effect of A5P in PEP+A5P reaction.

CO_2 evolution represents the value of CO_2 evolved between 25 and 40 minutes in μl.

The effect of PEP was distinct when in combination with A5P, but similar acceleration was also observed in combination with ATP or ADP with the use of a crude preparation. The accelerating effect in combination with ADP was the most remarkable (Table I) and this may show that pyruvate kinase reaction with ADP to form ATP proceeds far more rapidly than that with A5P to form ADP. The ratio of the velocity of the reaction of ADP and PEP to that of A5P and PEP at the same molar concentrations

TABLE I

Effect of Phosphate Compounds on the Reaction which was carried out with Various Adenine Nucleotides as Substrate

Nucleotide	A5P			ADP			ATP				
Addition	None	PEP	CP	None	PEP	CP	None	PEP	CP	Acetyl phosphate	Carbamyl phosphate
CO_2 evolution (μl.)	3	134	18	82	342	206	187	347	258	183	190

Concetrations of all the added compounds were $10^{-3}M$ each. The reaction condition was the same as in Fig. 1.

was near 2.0, which was approximately constant at each purification step of the enzyme preparation.

To analyze the effect of PEP upon the system of ATP+PEP, the same treatment as in the experiment of Fig. 1 was carried out on this system as shown in Fig. 3. Curves 5 and 6 are the acceleration of PEP and ATP against a definite concentration of PEP and ATP, respectively. The curve 6 attains a maximal plateau over a certain concentration of PEP, whereas the curve 5 declines from its maximum over a definite concentration of ATP. These facts might suggest that PEP may produce the reactant, ATP, from the reaction product, A5P, or in other words, PEP acts as an ATP regenerator. On the other hand, the similar effect as above PEP was also observed to a lesser extent in the case of creatine phosphate but not in other energy-rich phosphate compounds (Table I). In addition, PEP did not alter the initial reaction velocity by its addition on the ATP-system (Fig. 4) and pyruvate also had no such effect. All these facts support the above assumption on the mechanism of the effect of PEP, which is certainly not specific to this compound.

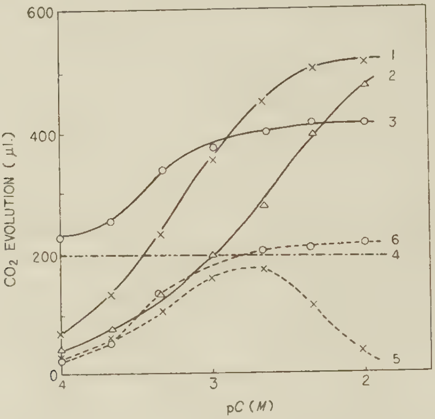

FIG. 3. Effect of PEP on ATP-system.

Curve 1 (—×—), activity of variant ATP when PEP was settled at 10^{-3} *M*; Curve 2 (—△—), activity of ATP alone; Curve 3 (—○—), activity of variant PEP when ATP was fixed at 10^{-3} *M*; Curve 4 (-·--·-), activity of 10^{-3} *M* ATP; Curve 5 (--×--), difference of the curves 1 and 2; Curve 6 (--○--), difference of the curves 3 and 4.

p*C*(*M*), -log value of molar concentration of specified substrate.

Of the ATP-regenerating systems tested the system of pyruvate kinase was found to be far more efficient than that of creatine phosphokinase. A striking difference between them was observed in the system of A5P as no detectable formation of TDP was found in the latter in contrast to the marked synthesis in the former (Table I). This finding might indicate that liver pyruvate kinase would take A5P as a direct substrate other than ADP without participation of the action of adenylate kinase. In fact, the treatment of the A5P preparation used here with *N* HCl at 100° for 15 minutes to decompose a possible trace of contaminant such as ATP and ADP in the preparation brought about no change in its attitude to the pyruvate kinase system as a phosphate donor. This fact led to the same conclusion as above, in other words, the activity of pyruvate kinase with A5P does not need concomitant presence of ADP or ATP. The effectiveness of A5P as a substrate to pyruvate kinase was reported by other workers in the enzymes derived from other sources (*9*, *10*), but the mechanism of its action remains yet unsettled whether it originates from the characteristic nature of this enzyme itself or the intermedialy formation of ADP from A5P by the aid of adenylate kinase. A tentative assumption of the presence of adenylic acid transphosphorylase which may catalyze the reaction, A5P+PEP → ADP+pyruvate, besides the usual pyruvate kinase to transfer phosphate from PEP to ADP to form ATP was made in a muscle preparation without evidence (*9*). The maintainance of constant activity ratio of ADP/A5P through the purification process in the present enzyme preparation might show an identity of the enzyme in liver corresponding to the two steps, but the final conclusion must await further investigation. The reason why the pyruvate kinase system is the most efficient accelerator for the activity of thiaminokinase may be attributed to the removal of harmful product, A5P, other than ADP, turning it into the substrate, ATP.

(*B*) *The Cause of Early Cessation of the Reaction*

As has already been shown in the ATP-reaction, the TDP-formation ceased almost completely after incubation of about 150 to 180 minutes with a small yield of TDP, for instance, 1–3 per cent of the reactant, thiamine, under the standard condition described in the previous paper (*2*). Now the extent of duration of the reaction was observed in the system with sufficient additon or absence of PEP to a definite concentration of ATP. The reaction proceeded linearly in the presence of PEP in spite of comparative early cessation in the system with ATP alone (Fig. 4). The continuance of the reac-

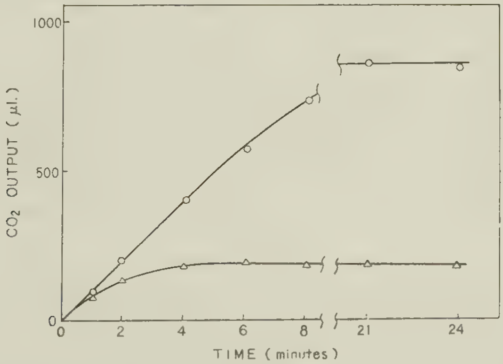

FIG. 4. Effect of PEP on the time course of long incubation. —△—, ATP (10^{-3} *M*); —○—, ATP (10^{-3} *M*)+PEP (2×10^{-3} *M*).

tion in the presence of PEP would reach even ten hours with the yield of TDP of about 20 per cent of thiamine added. Accordingly, it is certain that the cause of cessation of the ATP-reaction is not the inactivation of the enzyme. The addition of PEP or ATP anew to the system when the reaction had practically ceased resulted in a complete recovery of the activity

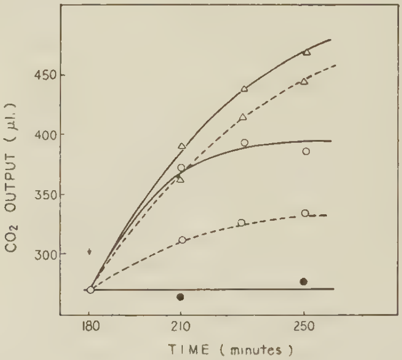

FIG. 5. Effect of PEP and ATP on the reaction after its cessation through incubation for 180 minutes under the standard condition as described in Fig. 1.

Arrow shows addition of the following materials: —△—, PEP (4.3×10^{-3} *M*); —○—, ATP (4.3×10^{-3} *M*); --△--, PEP (8.6×10^{-4} *M*); --○--, ATP (8.6×10^{-4} *M*); —●—, No addition.

(Fig. 5). Here the reaction with only ATP ceased some time later, and the level of TDP produced anew differed with the concentration of ATP added. On the contrary, in the case PEP was added, the reaction extended linearly and the reaction velocity was almost the same despite the different concentrations of PEP added. This fact shows again that the cause of cessation in the ATP-system is not inactivation of the enzyme itself.

The cause of cessation of the reaction in general can be classified as follows: 1) inactivation of the enzyme, 2) arrival to equilibrium point, 3) degradation of reactants, 4) inhibition by reaction products, and 5) other miscellaneous causes. According to observations made so far, the possibility of 1) and 2) could be excluded, and 3) and 4) may be considered as the possible causes.

It is clear that the reaction product, A5P, acts as an inhibitor and inhibition of the reaction would possibly be enhanced by the production of A5P and ADP from ATP through the action of adenylate kinase contained in the preparation (*2*). The theoretical production of A5P in the reaction of thiaminokinase could be assumed to be so small that the cessation could not be attributed to inhibition of the A5P formed by the reaction, but cessation of the reaction may be attributed mainly to the possible combining effect of adenylate kinase and adenosinetriphosphatase which may contaminate also the present preparation (*3*). In any case, decrease of ATP was remarkable during the reaction and velocity of the formation of TDP dropped roughly parallel to the content of ATP (Fig. 6). A slight discrepancy between them may be attributed to inevitable inhibition of the product, A5P. The product inhibition caused by TDP was excluded by an experiment showing no depression of the activity on addition of 3 μg. of TDP to the reaction mixture. Other possible causes of the cessation, such as degradation of the reactant, thiamine, and of the product, TDP, was disclaimed previously (*2*). Weil-herbe suggested in his earlier work (*1*) that the cessation of the reaction in yeast may be caused by the saturation of apocarboxylase with TDP, but in the present system apocarboxylase could not be detected in the preparation, and further no effect was observed on addition of pyruvic oxidase to the system.

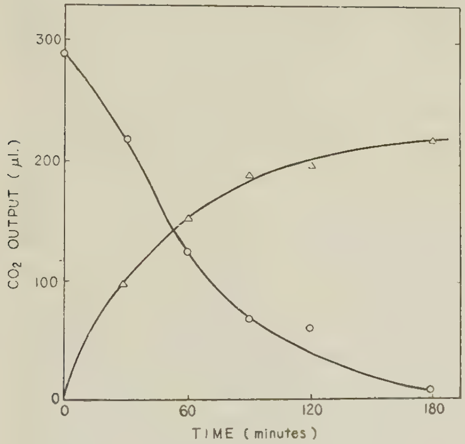

FIG. 6. Correspondence of decrease of ATP to cessation of the reaction.

—○—, Content of ATP; —△—, Formation of TDP.

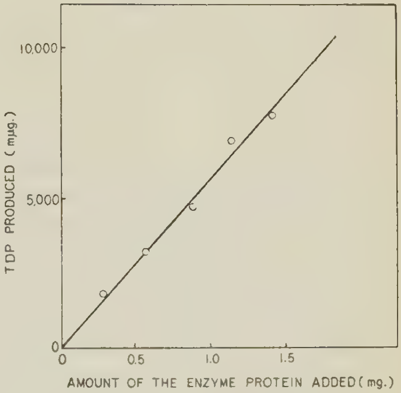

FIG. 7. Linear relationship between the initial velocity and amount of the enzyme.

Reaction mixture contained: 5×10^{-4} *M*, thiamine; 10^{-3} *M*, ATP; 3.3×10^{-3} *M*, $MgCl_2$; 2.8×10^{-2} *M*, Tris buffer of pH 8.0; and enzyme as specified. Total volume, 3.0 ml. The enzyme used was the purified preparation from the Step 6 in Part III of this series (*3*). Incubation, 15 minutes at 37°.

On the other hand, the absolute amount of TDP synthesized was, of course, influenced markedly by the amount of thiaminokinase incubated. The initial velocity changed proportionally with the amount of the enzyme added when other reactants were present in sufficient quantity (Fig. 7), but duration of the reaction could not be altered seriously with the amount of the enzyme. Therefore, the amount of the enzyme determines the yield of TDP but this cannot explain the cause of the cessation of the reaction.

(*C*) *Conclusion*

Upon considering the above experiments, the most plausible cause of the cessation is sluggish nature of the reaction, and other rapid interfering reactions decrease the reactant, ATP, to form the inhibitory products, ADP and A5P. Of the ATP-regenerating system under the system of pyruvate kinase was the most efficient in the present enzyme preparation by the conversion of inhibitory product to the reactant, A5P→ATP. Of course, the biosynthesis of TDP *in vivo* will occur with a number of other reactions, but there may be no such special regulating mechanism on the formation of this coenzyme as suggested by Weil-Malherbe (*1*). Since the level of various nucleotides whereby the production of TDP would be controled *in vivo* may be in dynamic equilibrium, some factors other than those indicated above might operate in intact system to affect the TDP formation.

SUMMARY

External factors which could influence the thiaminokinase reaction were investigated enzymatically with the preparation from rat liver, and the cause of cessation of the reaction in partially purified enzyme was discussed. The reaction is characterized by its sluggish nature and other rapid reacting enzymes which contaminate the preparation such as adenosinetriphosphatase and adenylate kinase would exhaust the phosphate donor, ATP, and increase the inhibitor, ADP and A5P. Thus the velocity of the interfering enzymes would overcome that thiaminokinase and led to the cessation of the reaction. Other possible causes of the cessation had all been excluded under the condition studied. The striking effect of acceleration by PEP on the reaction may arise from removal of A5P in addition to regeneration of ATP.

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ENZYMATIC ACTIVATION OF FORMIC ACID

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It has been demonstrated that formic acid is used as a one-carbon unit during reaction with tetrahydrofolic acid (THFA) and ATP. In this reaction, ATP is split to ADP and inorganic orthophosphate (*1*, *2*). According to the results of Whiteley *et al.* (*3*), activation of formic acid by a bacterial enzyme is preceded by the phosphorylation of the N^{10} of THFA by ATP.

On the other hand, recent studies on fatty acid activation revealed activation of the carboxyl group, forming acyl-phosphate in bacteria and acyl-AMP in animals and in yeast. This suggests a possibility that formic acid might also be activated in the same way as other fatty acids. The present communication is concerned with purification and some properties of a formate activating enzyme of this type isolated from *E. coli*.

The formate activating enzyme was prepared from acetone-dried cells of *E. coli*, strain B. The purification procedure involved the following steps: (1) Overnight extraction from acetone-dried cells with distilled water; (2) first precipitation with acetone (40-60 per cent); (3) removal of inactive proteins with calcium phosphate gel in $2\times10^{-2}\,M$ phosphate buffer at pH 7.2; (4) ammonium sulfate fractionation (53–60 per cent saturation); (5) second precipitation with acetone (50–60 per cent). The specific activity usually rises 35–45-fold at the final stage (step 5). The activity of the enzyme was measured spectrophotometrically by hydroxamic acid formation by the method of Schweet (*4*).

The activation of formate is strikingly dependent upon the presence of ATP. ATP can partially be substituted by GTP, but the explanation of the role of GTP must await further experiments using enzyme preparations of higher degrees of purity. Other nucleotides such as UTP, ADP and AMP were found to be inactive.

The crude extract shows little activity towards formate even in the presence of ATP. However, the addition of manganese enhances greatly the activity. The optimal concentration of this cation is $6.67\times10^{-3}\,M$. Of the other divalent cations tested, only magnesium was effective showing a slight activation (one-forth or one-fifth of that of the manganese) at the same concentration as the manganese.

During the purification of the enzyme, especially at step 4, considerable loss of the activity was observed. This inactivation, however, can be completely restored by addition of reduced glutathione at a final concentration of

$1 \times 10^{-3} M$. Cysteine, thioglycolate and 2-mercaptoethanol can also replace glutathione. In accordance with the above observation, it was found that the SH-blocking agents such as *p*-chloromercuribenzoate and heavy metals strongly inhibited the reaction of a crude extract which does not require SH-compounds for maximal activity. The inhibition is fully reversed by addition of the SH-reagents listed above.

A summary of these experiments is given in Table I.

TABLE I

Cofactor Requirements for Formate Activation

Reaction system	Hydroxymate formed (μ mole/20 minutes)
Complete system	1.76
— ATP	0
— $MnCl_2$	0.06
— $MnCl_2+MgCl_2$ (20 μ moles)	0.46
— glutathione	0.36
— glutathione+L-cysteine (5 μ moles)	1.27
— glutathione+thioglycolate (5 μ moles)	0.94

The complete system contained ; 600 μ moles of potassium formate, 15 μ moles of ATP, 3 μ moles of reduced glutathione, 20 μ moles of manganese chloride, 2 m moles of neutralized hydroxylamine, 150 μ moles of Tris buffer, pH 7.2, 136 μg. of formate activating enzyme (purification step 5) and water to give a final volume of 3 ml. The pH of reaction mixture was 7.2. All incubations were carried out at 37° for 20 minutes.

The pH range of the enzyme activity is wide, extending from pH 6.7 to 8.5 with an optimum at about 6.9.

Substrate specificity has not yet been determined, because our preparation was still contaminated with acetokinase. However, results suggest that the formate activating enzyme differs from the acetokinase (*5*).

Treatment of the enzyme by charcoal or Dowex-1 (Cl^- form) causes no appreciable loss of activity. This means that neither coenzyme A nor THFA participate in the formate activation in our system.

The product of the formate activation reaction in the presence of hydroxylamine was identified as formyl hydroxamic acid by paper chromatography. The reaction was stopped by the addition of perchloric acid at a final concentration of 5 per cent. After centrifugation and neutralization with potassium hydroxide, the supernatant was evaporated to dryness under reduced pressure. The product was taken up in a small volume of methanol and applied on Toyo-Roshi No. 51 filter paper. The hydroxamate was detected by spraying the paper with 5 per cent ferric chloride in 0.1 N hydrochloric acid. The solvent systems used for chromatography of the hydroxamate were as follows: (1) Butanol saturated with water ($R_f=0.43$); (2) tertial-amyl alcohol saturated with water ($R_f=0.61$); (3) butanol : formic acid : water (4 : 1 : 1) ($R_f=0.46$); (4) butanol : methylethylketone : ammonia : water (5 : 3 : 1 : 1) ($R_f=0.11$);

(5) butanol : ammonia : water (5 : 1 : 1) (R_f=0.04). These R_f values are consistent with those of synthetic formyl hydroxamic acid.

When the incubation was carried out in the absence of hydroxylamine, an unknown compound accumulated. This reacted with hydroxylamine forming formyl hydroxamate and was not adsorbed on charcoal. In addition, the accumulation of ADP was also demostrated after the incubation by the method of Magasanik *et al.* (*6*). Therefore it seems likely that the actual intermediate in formate activation is formyl phosphate rather than formyl adenylate.

Formate activating enzyme was also found in *Pseudomonas fluorescens* and *Aerobactor aerogenes.*

Identification of the intermediate, further purification of the enzyme and the physiological significance of the reaction are now being investigated.

After this manuscript was submitted, an enzyme, which also named as formate activating enzyme, was reported by H. R. Whiteley, M. J. Osborn and F. M. Huennekens (*J. Biol. Chem.*, **234**, 1538, 1959). This enzyme, however, has an entirely different mechanism for activating formate from that of ours. In order to avoid a confusion, the authors feel that it would be better to specify our preparation and to propose the name "formokinase" for it.

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STUDIES ON BACTERIAL AMYLASE

II. AMINO-TERMINAL AMINO ACID OF BACTERIAL AMYLASE

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(Received for publication, July 20, 1959)

A number of α-amylases have been obtained in crystalline form and their amino acid compositions (*1–5*) were also reported. However, studies on the chemical structure have been performed only on Taka-amylase A. Akabori and his coworkers reported that the N-terminal amino acid (*6*) of Taka-amylase A was alanine. They also studied on C-terminal amino acids (*7*), N-terminal peptide (*8*, *9*) and the structure of the peptide part combined with an oligosaccharide (*10*).

The present paper deals with the studies on the N-terminal amino acid of bacterial amylase (BA) by the 1-fluoro-2,4-dinitrobenzene (FDNB)-method (*11*) and the result is compared with N-terminus of Taka-amylase A.

EXPERIMENTALS

Bacterial Amylase—The crystalline BA was kindly supplied by Dr. B. Hagihara. This enzyme was crystallized from a culture medium of *Bacillus subtilis N* by Hagihara's method (*12*) and recrystallized several times.

DNP–BA—To 300 mg. of BA dissolved in 20 ml. of 2 per cent $NaHCO_3$ solution, 0.5 ml. of FDNB in 40 ml. of ethanol was added and the mixture was shaken in the dark for four hours. The dinitrophenyl (DNP)-protein precipitated by acidification (Thymol blue-red) of the mixture with concentrated hydrochloric acid, was centrifuged and washed six times with water, four times with ethanol and twice with ether. Then, DNP-BA was dried in vacuo over calcium chloride and ground in a mortar. The amount of original protein in the DNP-BA was estimated by the determination of the amide content of the DNP-enzyme, according to Bailey's (*13*) and Okada's (*14*) method. The amide content is shown in Table I.

Silica Gel—This was prepared in a similar method as described previously (*15*).

Identification of N-Terminus—About 1 μmole of DNP-BA was hydrolyzed with 3 ml. of distilled hydrochloric acid in a sealed tube at 105–110° for 5, 8, 15 and 24 hours. The hydrolysate was diluted with 6 ml. of water and DNP-N-terminus was extracted with ether. After the ether phase was washed with dilute hydrochloric acid, it was dried up. The residue was dissolved in 3 ml. of ethylacetate and trisected. Each fraction was dried in a desiccator and used for identification and determination of the N-terminus of BA by silica gel and celite column chromatography. The band corresponding to DNP-valine was cut, eluted with ethylacetate and methylalcohol, and organic solvent was removed. After the residue was dissolved in 5 ml. of 1 per cent $NaHCO_3$ solution, the

TABLE I

The Amide Content of DNP-BA

Experimental No.	Amount	Liberated NH_3	Amide NH_3
1*	2.426 *mg.*	24.3 (γ)	1.00 (%)
2*	3.700	38.9	1.05
3**	29.66	318	1.07

* Okada's method.

** Bailey's method.

optical density of this solution was measured at about 430 mμ by A.K.A. photo-electric colorimeter.

Recovery Factor of DNP-Valine during Acid Hydrolysis—A proper amount of DNP-valine was heated with distilled hydrochloric acid in a sealed tube for 5, 8, 15 and 24 hours. After the hydrolysate was diluted three fold with water, DNP-valine was extracted with ethylacetate and chromatographed on a silica gel column. The band of DNP-valine was extracted with ethylacetate and the organic solvent was expelled. The DNP-valine was estimated colorimetrically in 1 per cent $NaHCO_3$ solution. Table II shows recovery per cent for each acid hydrolysis.

TABLE II

Recovery of DNP-Valine during Acid Hydrolysis

Time of hydrolysis	Recovery per cent
5 (*hours*)	79 (%)
8	70
15	67
24	56

RESULTS AND DISCUSSION

Estimation of N-Terminal Amino Acid of BA—The chromatograms of the N-terminal fraction and various DNP-amino acids are shown in Fig. 1.

From Fig. 1 it is apparent that the N-terminal amino acid of BA is valine. The results of the quantitative determinations are summarized in Table III.

From the results described above it could be concluded that valine is a sole N-terminal amino acid of BA. Confirmation of N-terminal valine was made by regenerating free valine by treating with saturated barium hydroxide. Thus, it is shown that N-terminus of BA is different from that of Taka-amylase A (alanine). Therefore, it might be inferred that the N-terminal amino acids of α-amylases can not play an important role in the enzyme action.

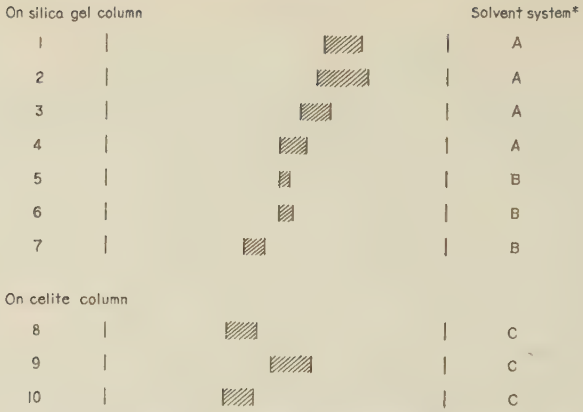

FIG. 1. Chromatograms of N-terminal fraction and various DNP-amino acids.

1,5,8	N-terminal fraction of DNP-BA	6,10	DNP-Val
2	DNP-Val,-Leu	7	DNP-Met
3	DNP-Phe	9	DNP-Leu
4	DNP-Pro,-Ala,-Met, di-DNP-Tyr,-Lys.		

* A: chloroform-20 per cent acetic acid.
B: 1 per cent butanol, chloroform-10 per cent acetic acid.
C: 20 per cent methylethylketone, chloroform—pH 6.3 $M/10$ phosphate buffer.

Note: The Rc** values of the DNP-Asp, Glu, Ser, Thr, Gly and -Ala are less than 0.5 on a silica gel column using the solvent system A.

** : Rc value expresses the ratio of moving distance of DNP-amino acid to that of the front of developing solvent.

TABLE III

Quantitative Determination of N-Terminal Valine

Experimental No.	DNP-BA	BA	Time of hydrolysis	DNP-valine*	Moles of DNP-valine per mole of BA**
	mg.		*(hours)*	*(µmoles)*	*(moles)*
1	52	42.4	5	0.68	0.78
2	50	40.8	8	0.81	0.97
3	56	45.7	15	0.86	0.92
4	52.5	42.8	15	0.76	0.87
5	52	42.4	18	0.76	0.87

* These values were corrected for destruction during acid hydrolysis.
** The molecular weight of 4,8700 (*5*) was used for calculation.

SUMMARY

The identification and quantitative determination of N-terminal amino acid of bacterial amylase (*B. subtilis N*) were performed by FDNB-method.

It was shown that valine was a sole amino-terminal amino acid.

The author wishes to express his thanks to Prof. S. Akabori and to Mr. Y. Okada for their kind guidance throughout this investigation and also to Dr. B. Hagihara for the gift of crystalline bacterial amylase.

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STUDIES ON DENITRIFICATION

IV. PARTICIPATION OF CYTOCHROMES IN THE DENITRIFICATION*

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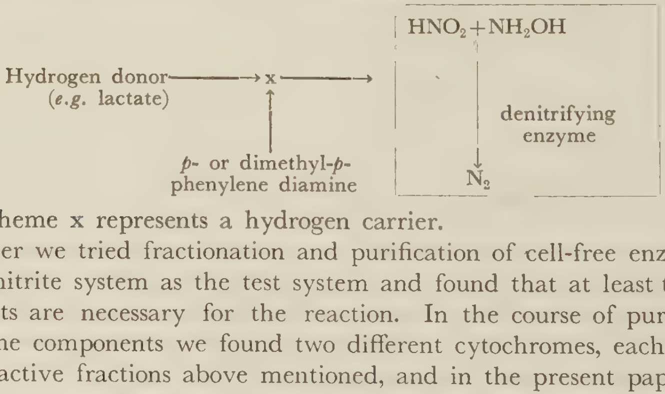
(Received for publication, July 25, 1959)

In the previous reports (*1*, *2*) we showed that a strain of denitrifying bacteria utilizes *p*-phenylene diamine and dimethyl-*p*-phenylene diamine (DMPPD) as hydrogen donors, that is, when we give sodium nitrite as hydrogen acceptor together with one of these compounds, for instance, dimethyl-*p*-phenylene diamine as hydrogen donor to the bacteria at neutral pH, nitrogen is produced enzymatically in the anaerobic condition. In this case the quantity of nitrogen evolved was about twice as that of nitrite-nitrogen. The bacterial enzyme was also shown to be able to catalyze the reaction of nitrite and hydroxylamine at neutral pH, producing nitrous oxide. As for mechanism of ordinary denitrification in which nitrite is converted into gaseous nitrogen, receiving electrons from a reducing system such as lactate-lactic enzyme system, we suggested that nitrite is reduced to an amine, the amine reacts with remaining nitrite, producing nitrogen, and as such an amine hydroxylamine was considered to be a possible intermediate in the ordinary denitrifying process. The assumed scheme is shown as follows:



In this scheme x represents a hydrogen carrier.

Further we tried fractionation and purification of cell-free enzyme using DMPPD-nitrite system as the test system and found that at least two protein components are necessary for the reaction. In the course of purification of the enzyme components we found two different cytochromes, each in one of the two active fractions above mentioned, and in the present paper we describe the method of purification starting from cell-free extract, natures and activities of the cytochromes, and offer a possible scheme of denitrification.

MATERIALS AND METHODS

The microorganism used is the same as described in the previous paper, namely a heterotrophic, facultatively anaerobic bacterium, isolated from soil. Although the identification has not yet been carried out, it is inferred to be a strain of *Pseudomonas denitrificans*.

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* Most parts of the work were presented in the Annual Meeting of the Japan Botanical Society, Nov. 1958 at Fukuoka, Kyushu.

Six loopfuls of bacteria were inoculated into 60 ml. peptone-meat extract-0.5 per cent KNO_3 medium at pH 6.8. After 24 hours' incubation the whole culture was inoculated into 6 liter medium and this preculture was incubated for 48 hours. Finally the 6 liter culture was transferred to a tank which contained sterilized 50 liter medium in advance, and after about 24 hours' incubation the bacterial mass was collected using a Sharples centrifuge at low temperature. All culture was carried out at 30° in static condition. The harvested bacteria were lyophilized using cold acetone by a common procedure, *e.g.* described in (*3*).

The method of estimation of gas evolution was the same as described in the previous paper, *viz.* in the nitrogen atmosphere at pH 6.8 at 30° using a Warburg manometer (*2*).

Dimethyl-*p*-phenylene diamine dihydrochloride and hydroxylamine hydrochloride were neutralized with sodium hydroxide immediately before use.

Absorption spectra were determined by a Beckman DU-type spectrophotometer. For the observation of spectra in the visible region Zeiss microspectral ocular was used, too.

Cytochrome oxidase of beef heart was obtained by the usual method, though somewhat modified. From fresh heart muscle particle suspension in phosphate buffer was obtained by centrifugation. The pH of the cold suspension was adjusted to 5.6 by addition of cold 1 *M* acetic acid and then centrifuged. The precipitate was suspended in phosphate buffer (*M*/10, pH 7.5). Cytochrome oxidase in the preparation was then, precipitated by addition of ammonium sulfate up to 0.5 saturation, separated on centrifuge and suspended in phosphate buffer.

Nitric oxide was prepared from acidified ferrous sulfate and sodium nitrite (*4*). The study of the reaction of cytochromes to nitric oxide was carried out in the complete absence of oxygen in Thunberg tubes, that is, in advance the tube was evacuated, washed several times with pure nitrogen to remove oxygen completely, and then it was filled with pure nitric oxide.

Purification of Denitrifying Enzymes and Cytochromes—Acetone powder of denitrifying bacteria was extracted with *M*/20 phosphate buffer at pH 6.8 and cell-free extract was obtained by centrifuging at $5{,}000 \times g$ for 15 minutes. This extraction was repeated three times. The combined extract was centrifuged at $20{,}000 \times g$ for 10 minutes. The supernatant was fractionated with ammonium sulfate into two parts; the fraction *A* between 33 and 45 per cent saturation of ammonium sulfate and the fraction *B* between 45 and 70 per cent saturation. If one may have desire to obtain only one active enzyme fraction for some purposes, the most active fraction can be separated between 40 and 50 per cent saturation of ammonium sulfate.

After repeating fractionation several times on each fraction, fraction *A* and *B* alone showed no gas producing activity, but when both were combined, a remarkable gas production took place with DMPPD-nitrite system. Each fraction was, then, dialyzed against distilled water in a cellophane tube for more than 7 hours. The dialyzed enzyme solution was each passed through a column of cation exchange resin, Amberlite CG-50 equilibrated at pH 6.0.

The active protein in the fraction *A* passed through the column without being adsorbed. The enzyme was collected by precipitating at 60 per cent saturation of ammonium sulfate and centrifuging at $8{,}000 \times g$ for 10 minutes. The precipitate was dissolved in *M*/20 phosphate buffer at 6.8 and dialyzed against the same buffer. This fraction *A* had a yellowish red color which is ascribable to the presence of a cytochrome component, which should be named cytochrome c_1^{552} (denitrifying bacteria).*

In flowing the column of the fraction *B*, the upper part of the column of Amberlite CG-50 adsorbed a red pigment, and on the lower part of the column, a blue pigment was adsorbed. When they were eluted by *M*/2 phosphate buffer at pH 6.8, the blue protein flowed down at first and the red pigment followed. The red pigment was named cryptocytochrome c for the reason mentioned in the other part of this paper (see p. 176 f) and this fraction *B* revealed denitrifying activity in combination with the cytochrome c_1^{552} fraction.

All treatments were carried out at low temperature.

Oxidation-reduction potential of cytochrome c_1^{552} was measured by the method of Davenport and Hill, using ferrocyanide and ferricyanide as oxidation-reduction buffer (*5*).

RESULTS

Denitrifying Activity of Fraction A and B with DMPPD-nitrite System—As shown in Fig. 1, neither fraction *A* nor *B* manifests gas production at all,

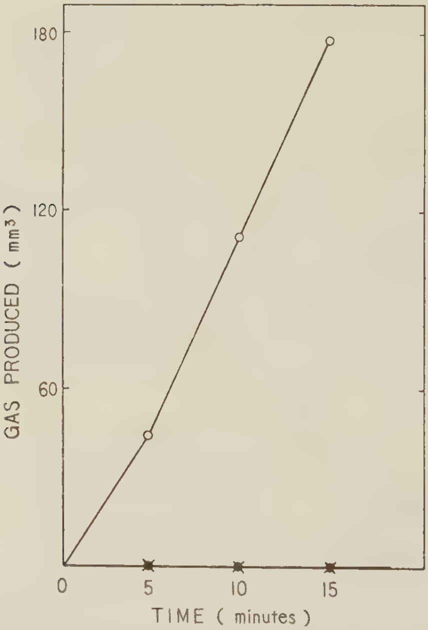

FIG. 1. Gas production from dimethyl-*p*-phenylene diamine and nitrite by the system of cytochrome c_1^{552} and cryptocytochrome c.

Phosphate buffer *M*/2 pH 6.8 0.3 ml., dimethyl-*p*-phenylene diamine *M*/2 0.3 ml., cryptocyt. c 0.1 ml. or cyt. c_1^{552} 0.2 ml. or both, in the main compartment.

NaNO$_2$ *M*/22.4 0.3 ml. in the side arm.

20 per cent KOH 0.2 ml. in the center well.

Nitrogen in the gas phase. Total volume: 3.2 ml.

—○— cryptocyt. c+cyt. c_1^{552}; —●— cryptocyt. c; —×— cyt. c_1^{552}.

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* The representation of the name of cytochrome components follows the formalities given in "Provisional Proposals" (*6*).

but if the both are added at the same time, strong gas production is observed, indicating that two components are involved in the gas producing system of nitrite and DMPPD.

The Nature of Cytochrome c_1^{552} *(denitrifying bacteria)*—Fraction *A* is colored reddish yellow. Absorption spectra of the cytochrome are shown in Fig. 2, representing typical absorption spectra of c-type cytochrome. The absorption maxima lie at 552 mμ, 523 mμ and 418 mμ in the reduced form and 408 mμ in the oxidized form. The hemin moiety cannot be splitted by the usual HCl-acetone treatment. Addition of pyridine and reducing agent shifts the absorption maxima about 2 mμ toward blue, that is, the pyridine hemochromogen has the α band at 550 mμ. From these evidences we can conclude that the cytochrome concerned is a cytochrome of c-type. The cytochrome cannot, however, replace cytochrome c of heart muscle in the oxidation of hydroquinone with heart muscle cytochrome oxidase, or it does not link with cytochrome oxidase prepared from beef heart. Though the reduced form is oxidized in the air slowly, this oxidation stays away entirely in the presence of 10^{-4} diethyldithiocarbamate, indicating that the cytochrome is not autooxidizable.

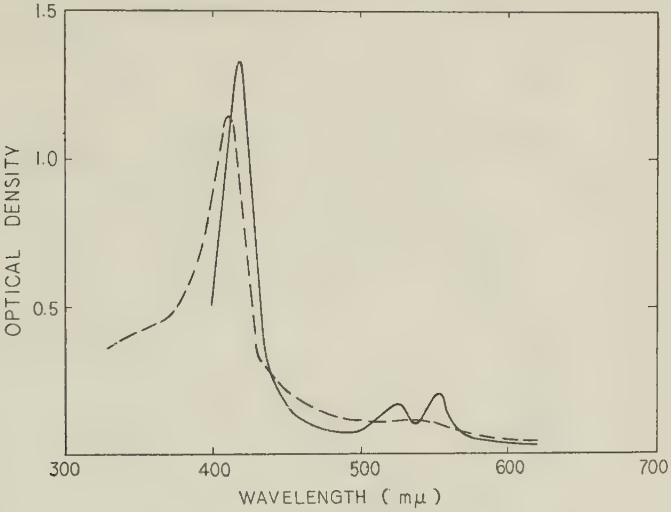

FIG. 2. Absorption spectra of cytochrome c_1^{552} (denitrifying bacteria) at pH 6.8. Light path: 1 cm. Broken line, in oxidized form; solid line, in reduced form.

The Nature of Cryptocytochrome c—The color of the fraction *B* is red and is due to cryptocytochrome c present. Even in the presence of sodium dithionite we can recognize no distinct α and β band in the visible spectral region (Fig. 3), but a broad weak absorption from about 540 to 560 mμ and a sharp band at 423 mμ, having a small shoulder at 430-440 mμ in the reduced form, and a corresponding γ band at 394 mμ in the oxidized form. On addition of a small amount of pyridine very sharp bands appear at 551 mμ, 523 mμ and

412 mμ in the reduced form. The hemin part cannot be splitted by the HCl-acetone treatment, and by this treatment, after neutralization, the cyto-

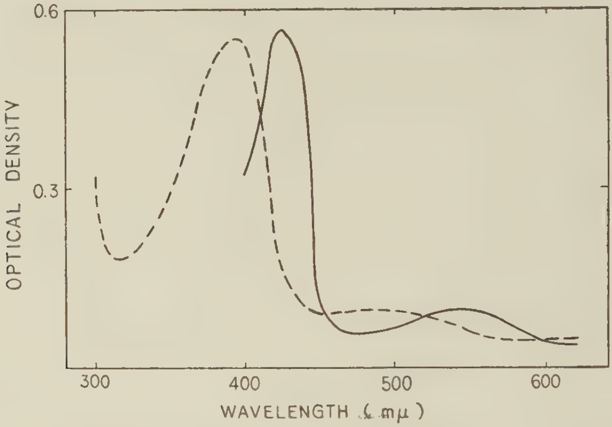

FIG. 3. Absorption spectra of cryptocytochrome c (denitrifying bacteria) at pH 6.8. Broken line, in oxidized form; solid line, in reduced form.

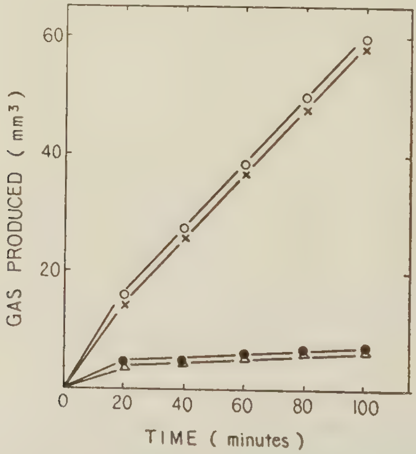

FIG. 4. Gas production from hydroxylamine and nitrite by cryptocytochrome c.

Cyt. c_1^{552} 0.2 ml. or cryptocyt. c 0.2 ml. or both, phosphate buffer *M*/2 pH 6.8 0.3 ml., $NaNO_2$ *M*/22.4 0.3 ml. in the main compartment.

Hydroxylamine *M*/2 0.3 ml. in the side arm.

20 per cent KOH 0.2 ml. in the center well.

Nitrogen in the gas phase. Total volume: 3.2 ml.

—○— cyt. c_1^{552}+cryptocyt. c; —×— cryptocyt. c; —●— cyt. c_1^{552}; —△— no cytochrome.

chrome is altered to show sharp absorption bands in the presence of sodium dithionite, as a remarkable fact, without pyridine, namely absorption bands at 550 mμ, about 520 mμ and 416 mμ in the reduced state. In the oxidized state the corresponding γ band is at 409 mμ. It means that typical absorption bands of c-type cytochrome appear by the HCl-acetone treatment. For this reason we named it cryptocytochrome c. Such an alteration of the optical property is, however, observed neither by a treatment with dilute hydrochloric acid nor by a treatment with alkali, nor by the action of solvents, acetone, ether and both in the mixture. Cryptocytochrome c does not link with beef heart cytochrome oxidase, too.

Gas Production with Nitrite-Hydroxylamine System by the Cryptocytochrome c Fraction—We reported in the previous paper (*2*) that nitrite reacts enzymatically with hydroxylamine producing nitrous oxide. This gas production can be realized by the fraction *B* alone, as shown in Fig. 4. Owing to the spectroscopic data described later, we suppose that this reaction is catalyzed by cryptocytochrome c itself, as shown in the following scheme:

$$HNO_2 + NH_2OH \xrightarrow[\text{cryptocyt. c}]{} N_2O + 2H_2O$$

The N_2O production catalyzed by the enzyme of fraction *B* is by no means inhibited by the presence of 20 per cent oxygen.

Replaceability of Cytochrome c_1^{552} (denitrifying bacteria) by other Cytochromes obtained from other Bacteria—(*1*) *Cytochrome c_1^{552} (Acetobacter suboxydans)*: A strict aerobic bacterium, *Acetobacter suboxydans* can oxidize lactate intensely and does not indicate any sign of reduction of nitrite, nitrate and hydroxylamine (*7*). From the bacterium a cytochrome of the similar optical properties to those of the denitrifyer cytochrome is obtained by the same method as that for the latter. The *Acetobacter* cytochrome was inferred to participate as hydrogen carrier in the lactate oxidation of the bacterium (*8*). When this cytochrome is combined with cryptocytochrome c fraction, gas production takes place with the DMPPD-nitrite system, although each, separately, shows no gas producing activity at all.

(*2*) *Cytochrome c_1^{552}* (halotolerant bacteria): A cytochrome component was obtained from acetone powder of a halotolerant bacterium, strain No. 203, Sb-type by extraction and purification through fractionation with ammonium sulfate and adsorption on the resin in the same way as the cytochrome of the denitrifyer. The cytochrome had similar optical and other properties to the latter.* Moreover, this cytochrome can substitute the cytochrome of denitrifying bacteria, particularly the activity is high in 10 per cent NaCl solution, indicating the halophilic nature of the cytochrome (9–11).

(*3*) Other cytochromes. Heart muscle cytochrome c (Sigma Chem. Co., U.S.) cannot substitute the cytochrome c_1^{552}.

A cytochrome of c-type can be obtained in a good yield, from the same halotolerant bacterium as mentioned above, by extraction with 50 per cent acetone, precipitation by addition of acetone and fractionation with ammonium sulfate.* The cytochrome obtained may be called cytochrome $c^{552,548}$ because of its double-peak α-band.** The cytochrome is, however, found not to replace the proper electron carrier in the denitrifying system.

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* The preparation of the cytochrome was given from late Dr. K. Hirai, Assistant Prof. of Nagoya City University, by his courtesy.

Oxidation-Reduction Potential of Cytochrome c_1^{552}—E_0' of cytochrome c_1^{552} (denitrifying bacteria) is found to be about +300 mv. at pH 7.0, when measured in the presence of 10^{-4} *M* diethyldithiocarbamate which is able to suppress the autooxidation of the reduced cytochrome in the air completely.

Absorption Spectra of Cryptocytochrome c in the Presence of Nitrite, Nitric oxide or Hydroxylamine—By an addition of nitrite (*M*/224) no appreciable change of spectrum is brought about in the oxidized state, but a remarkable change occurs in the absorption spectrum of reduced cryptocytochrome c, dissimilar to heart muscle cytochrome c, with which such a difference of spectrum could never be observed (Fig. 5, Table 1).

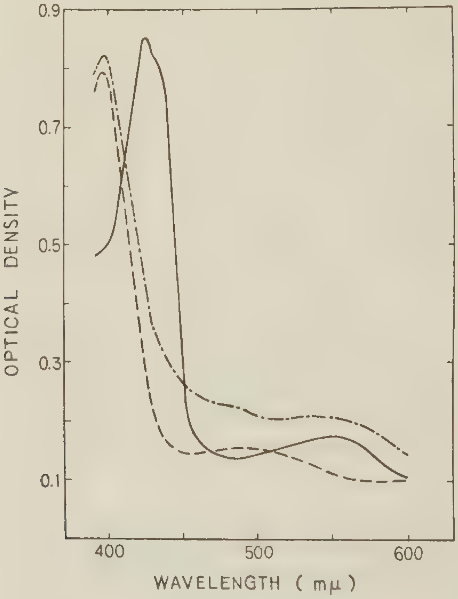

FIG. 5. The change of absorption spectrum of reduced cryptocytochrome c in the presence of nitrite or nitric oxide at pH 7.0.
— — — in oxidized state; —— in reduced state; —·—·— in reduced state in the presence of nitrite (*M*/224) or nitric oxide.

Absorption spectrum of cryptocytochrome c is changed by the presence of nitric oxide also only in the reduced state in the same way as seen by the presence of $NaNO_2$ (Fig. 5). This change is attributable to a combining reaction of nitric oxide with the cytochrome and in fact the reaction is found to be reversible. The effect of NO on heart muscle cytochrome c is different from that of $NaNO_2$, *viz.* it appears the NO-band in the oxidized state, as already known (see Table I). With the naked eye the pale pink color of reduced cryptocytochrome c changes into red by an addition of $NaNO_2$ or NO almost immediately.

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* The preparation of the cytochrome was also supplied by Dr. Hirai.

** The cytochrome has been studied originally by Dr. R. Sato (at present in the Institute for Protein Research, Osaka University) and written as cytochrome b_{4I} (*12*).

TABLE I.

The Change of Absorption Spectra of Cryptocytochrome c in the Presence of Nitrite or Nitric Oxide

	Heart muscle cytochrome c	Cryptocytochrome c
Oxidized state	407 (mμ)	394 (mμ)
Oxidized state + $NaNO_2$	407	394
Oxidized state + NO	417, 527, 563	394
Reduced state	415, 521, 550	423
Reduced state + $NaNO_2$	415, 521, 550	394
Reduced state + NO	415, 521, 550	394

On addition of NH_2OH ($M/20$) at pH 7.0 the reduced spectrum shows no change, while a small change is observed in the oxidized state of cryptocytochrome c, as shown in Fig. 6. Measurement was carried out immediately

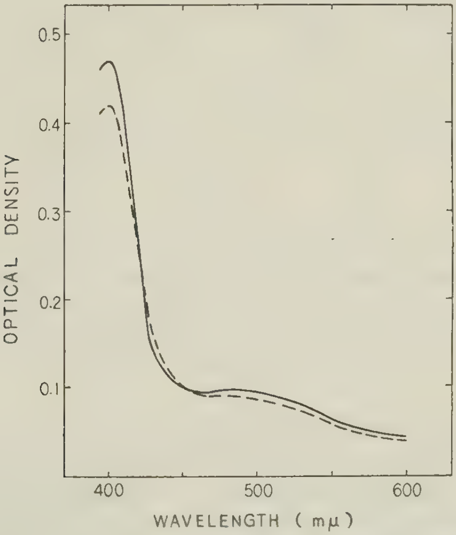


FIG. 6. Absorption spectra of oxidized cryptocytochrome c at pH 7.0 in the presence of hydroxylamine. Solid line, in oxidized state; broken line, in oxidized state in the presence of hydroxylamine ($M/20$).

after hydroxylamine was added, because such concentrated hydroxylamine was known to deprive the denitrifying activity within a short time, if nitrite was not present at the same time (*2*). Heart muscle cytochrome c is reduced slowly in such a high concentration of hydroxylamine used. This slow reduction is likely to be ascribed to the action of some decomposing products of neutralized hydroxylamine.

When both nitrite and hydroxylamine are added at the same time to the oxidized cytochrome, the same shape of absorption curve as that of the nitrite- or NO-complex of reduced cryptocytochrome c appeared (Fig. 7). The measurement was done immediately after the addition, because the preparation manifested very strong denitrifying activity with both components, NH_2OH and $NaNO_2$.

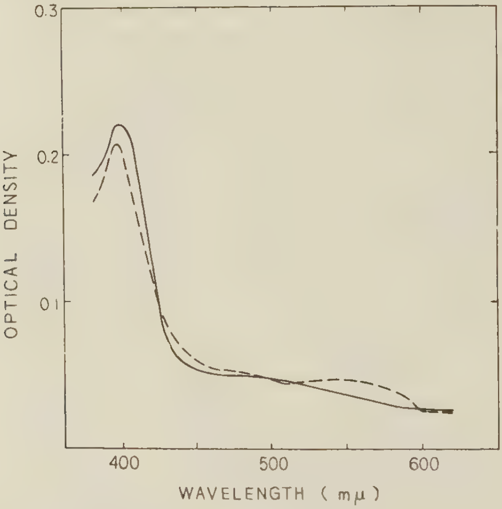


FIG. 7. The change of absorption spectrum of cryptocytochrome c (oxidized state) in the presence of nitrite and hydroxylamine at pH 7.0. Solid line, in oxidized state; broken line, in oxidized state in the presence of nitrite (*M*/112) and hydroxylamine (*M*/20).

Cyanide Dialysis of Cryptocytochrome c Fraction—In order to examine implication of some metal ions in the denitrifying activity (*1*), the cryptocytochrome c fraction was dialyzed against *M*/10 KCN at pH 7.2 for 7 hours and after the cyanide dialysis it was dialyzed against deionized water overnight. Activity was measured with NH_2OH-$NaNO_2$ system. Most part of the activity remains after this treatment. Furthermore, addition of some common heavy metal ions, *e.g.* Fe^{++}, Fe^{+++}, Mn^{++} and MoO_4^{--} (each in 2×10^{-4} *M*), can bring about no change in the denitrifying activity. By addition of Cu^{++} the rate of gas production increases. The effect is, however, due to pure chemical reaction of the inorganic ion catalyst, because the enhanced part of the reaction rate is just ascribable to the catalytic action of the free copper ion.

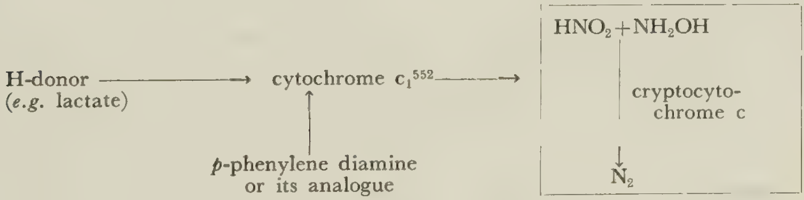
Further Purification of Cryptocytochrome c—Some efforts were made to examine the possibility that cryptocytochrome might be separated from the enzymic activity. Thus, fractionation was intended by using acetone, resulting that all the cryptocytochrome c is contained in the precipitate between 50 and 70 per cent concentration accompanying with the enzymic activity.

DISCUSSION

From the facts described above, cytochrome c_1^{552} is considered to be no specific electron carrier to the denitrifying system, but a general one which possesses also capacity to act as a necessary factor in the denitrifying system.

As for cryptocytochrome c, although we occasionally experienced an important fact that hemin content, as roughly judged from the intensity of color of solutions, is not always parallel to the denitrifying activity, the following facts summarized from the experimental results, would support the view that the denitrifying enzyme is cryptocytochrome c itself; 1. By the cyanide dialysis of cryptocytochrome c fraction the denitrifying activity is not reduced and addition of heavy metal ions has no effect on the activity. 2. The absorption spectrum of the reduced form is changed on addition of nitrite or nitric oxide and that of the oxidized form alters in the presence of both nitrite and hydroxylamine at the same time. 3. By acetone fractionation all the cryptocytochrome c together with activity is contained in a narrow fraction between 50 and 70 per cent concentration of acetone.

As a conclusion at the present stage, we should like to formulate the following scheme as a possible mechanism of the denitrifying system of our bacteria.



SUMMARY

1. Using dimethyl-*p*-phenylene diamine and nitrite as the test system bacterial denitrifying enzymes were purified. The cell-free extract obtained by centrifugation at 20,000 × *g* was fractionated with ammonium sulfate and cation exchange resin and two cytochromes were obtained separately, namely cytochrome c_1^{552} and cryptocytochrome c.

2. Cytochrome c_1^{552} is found to be a kind of typical c-type cytochrome, except that it does not link with heart muscle cytochrome oxidase. The E_0' value of the cytochrome is about +300 mv (pH 7.0).

3. Cryptocytochrome c shows no distinct α- and β-band in the reduced form, instead, a broad band between 560 and 540 mμ and a sharp band at 423 mμ, and 394 mμ in the oxidized state. Treatment with HCl- acetone does not effect a splitting of the hemin part, and after the treatment, on neutralization and reduction, sharp absorption bands appear which are reminiscent of those of a typical cytochrome c. The cytochrome does not link with heart muscle cytochrome oxidase, too.

4. The fraction containing cryptocytochrome c has N_2O producing activity from hydroxylamine and nitrite. Inhibition of the gas production by oxygen cannot be observed in this reaction.

5. Cytochrome c_1^{552} (denitrifying bacteria) is regarded as an electron carrier in the system of nitrite and dimethyl-*p*-phenylene diamine, and it can be substituted by cytochrome c_1^{552} (*Acetobacter suboxydans*) and c_1^{552} (halotolerant bacteria, strain No. 203, Sb-type).

6. Absoption spectrum of cryptocytochrome c is changed by an addition of nitrite or nitric oxide in the reduced state and a little by the presence of NH_2OH in the oxidized form. A remarkable change of the absorption spectrum was observed also by an addition of $NaNO_2$ and NH_2OH at the same time. It seems probable that cryptocytochrome c is the denitrifying enzyme itself.

7. From the experimental results obtained a possible scheme of denitrification is proposed.

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STUDIES ON THE RIBONUCLEOPROTEIN PARTICLES

VII. EFFECT OF VARIOUS HYDROGEN BOND FORMING AGENTS ON THE MICROSOMAL RIBONUCLEOPROTEIN PARTICLES

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As to the nature of the bonds which hold RNA and protein of ribonucleoprotein (RNP) together, two lines of evidence seem to have been accumulated. In the one, hydrogen bonds are assumed to be important, because RNA can be isolated from animal tissues by treatment with phenol (*1*), and also urea can effectively dissociate the RNP of *Escherichia coli* to RNA and protein (*2*). In the other, much importance is attached to ionic bonds, because the RNP isolated from rat liver microsomes was dissociated to RNA and protein in strong saline (*3*).

It seems important, therefore, to examine the effect of various hydrogen bond forming agents on the RNP at neutral pH in order to determine which view is more likely.

Hydrogen bond exists between a proton donor (D-H) and proton acceptor (A), forming an equilibrium $\text{D-H}\cdots\text{A} \rightleftarrows \text{D-H}+\text{A}$. According to the strength of the hydrogen bonds formed, proton acceptor and proton donor series have been reported (*4*, *5*, *6*). From these, various agents have been selected taking their solubility as an aqueous solution into consideration. As such agents, pyridine, picoline, phenol, *o*-cresol, *p*-nitrophenol, *etc.* were selected and their effects on the microsomal RNA were examined. As the effects of urea have already been reported (*7*), it was excluded from the present paper.

EXPERIMENTALS

Isolation of the RNP—The RNP was isolated from rat liver microsomes by the treatment with sodium deoxycholate as previously described (*8*, *9*). The deoxycholate insoluble fraction was dissolved in the Miller-Golder's buffer of ionic strength 0.1 and pH 7.0 (*10*), and centrifuged at $20,000 \times g$ for 20 minutes. The clear supernatants were carefully pipetted out and used as the purified RNP. The loosely packed sediments were also reserved to examine the effects of hydrogen bond forming agents.

Chemicals—Pyridine, phenol, 2,4-dinitrophenol, pyrocatechol, and resorcine were analytical grade, and α-picoline, *o*-cresol, salicylic acid, benzoic acid, *m*-aminophenol, and hydroquinone were extrapure reagents, and used without further purification. These reagents were dissolved in the buffer at a final concentration listed in the Table I, pH of the solution being always adjusted to 7.0 if necessary.

Effect of the hydrogen bond forming agents on the dissociation of the RNP were examined by paperchromatography, electrophoresis, or by centrifugal method.

Paperchromatography—Paperchromatography of the RNP was carried out in the similar manners as previously described, using these agents as solvent (*7*), RNA was located in ultraviolet light and protein was demonstrated by the staining with 0.05 per cent bromphenol blue.

Electrophoresis—Electrophoretic analysis was usually carried out in the buffered solution of hydrogen bond forming agent. To the RNP solution of which concentration was 20-15 mg. per ml., an aliquot of buffered solution of hydrogen bond forming agents was added in the cold. Then the samples were dialysed overnight in the cold against the corresponding buffered solution containing the same agent at an equal concentration, centrifuged at 2,500×*g* for 15 minutes in the cold if necessary, and then the samples or the supernatants were analysed electrophoretically in the same solution. Employing Hitachi Model HTD-1 electrophoretic apparatus electrophoretic analysis was carried out at 4°. Conductivity of the buffer solution containing these agents was measured at 0° by Wheatstone's bridge method and the electophoretic mobility at 0° was calculated.

Centrifugal method—As the protein moiety of the RNP is hardly soluble in neutral buffer (*11*), it is expected that the dissociation of the RNP by the hydrogen bond forming agents might result in the precipitation of protein if it is not solubilized by these agents. An aliquot of the buffered solution of the hydrogen bond forming agent was added to the RNP solution at a definite final concentration. Standing still overnight in the cold, the solution were centrifuged, and the supernatants were carefully pipetted out. RNA and protein both in the supernatant and in the sediments were determined by the following method.

Analysis of RNA and protein—The Schmidt-Thannhauser's method slightly modified by Littlefield *et al.* was employed throughout (*9*). RNA content of the samples was determined from the optical density at 260 mμ and protein content was determined by Lowry's method (*12*). In these determinations, however, much cares ought to be paid to eliminate disturbance due to these hydrogen bond forming agents completely, because, the agents may interfere either with ultraviolet absorption and/or with the Folin's reaction (*12*) and so following procedures were used. Phenol, *o*-cresol, *p*-nitrophenol *etc.* were effectively eliminated by three or four times extraction with ether, before the addition of cold perchloric acid, while pyridine, α-picoline *etc.* were extracted several times with alcohol-ether-chloroform mixture (2 : 2 : 1) after precipitation with cold perchloric acid. When saturated phenol or cresol was used, it is often difficult to remove these agents completely. As maximum absorption of the nucleotides mixture of RNA in 0.05 N perchloric acid was near at 260 mμ and that of phenol or of *o*-cresol was at 270 mμ, the amount of RNA was determined by dichromatic readings at 260 and 270 mμ.

RESULTS

In Table I are listed the hydrogen bond forming agents used in this experiment, together with their final concentration, macroscopic turbidity-increase of the RNP solutions after addition of these agents and their effect on the dissociation of the RNP to RNA and protein at pH 7.0.

I. Phenol and Its Derivatives

Phenol, which has been used to isolate RNA from tissues (*1*), is at the same time, known as one of the strongest proton donors (*4*, 6), and so it

TABLE I

A List of the Hydrogen Bond Forming Agents Which Were Used to Examine Their Effects on the Microsomal RNP Particles

Hydrogen bond forming compounds	Concentration[1]	Macroscopic turbidity increase[2]	Dissociation[3]
1) Phenol	3 %	(+)	(÷)
	6 %	(+)	(+)
2) *o*-cresol	1.2 %	(+)	(÷)
	2.4 %	(+)	(+)
3) *p*-nitrophenol	0.5 %	(+)	(÷)
	1.0 %	(+)	(÷)
4) 2,4-dinitrophenol	0.5 %	(−)	(−)
5) *m*-aminophenol	0.5 %	(−)	(−)
6) Pyrocatecol	2.5 %	(−)	(−)
7) Resorcine	2.5 %	(−)	(−)
8) Hydroquinone	2.5 %	(÷)	(−)
9) Benzoic acid	0.2 %	(−)	(−)
10) *p*-amino benzoic acid	0.2 %	(−)	(−)
11) Salicylic acid	0.2 %	(−)	(−)
12) Pyridine	5 % (*v*/*v*)	(−)	(÷)
	10 % (*v*/*v*)	(+)	(÷)
	20 % (*v*/*v*)	(+)	(÷)
13) *α*-picoline	20 % (*v*/*v*)	(+)	(÷)
14) Urea	2.0∼6.0 *M*	(÷)	(÷)

1) The figures in this column mean weight by volume percentage excepting pyridine and *α*-picoline of which concentration is represented by volume percentage. As the solvent of these solutions, the Miller-Golder's buffer of pH 7.0 and ionic strength 0.1 was used throughout.

2) (÷) means slight increase in turbidity of the samples after addition of the agents.

3) (÷) means that the agent was slightly effective to dissociate the RNP but was far less complete than 6 per cent phenol or 2.4 per cent *o*-cresol.

seems to be interesting to examine the effect of phenol and its derivatives such as *o*-cresol, *p*-nitrophenol, 2,4-dinitrophenol, and *m*-aminophenol, *etc.* on the microsomal RNP.

The latter two compounds caused no turbidity increase when added to the RNP solutions, and moreover, the electrophoretic patterns of the RNP were hardly influenced by these compounds (Fig. 4-a, b). Phenol, *o*-cresol, and *p*-nitrophenol, on the contrary, had remarkable effects on the RNP, their effects being exclusively examined here.

Electrophoretic Analysis of the RNP Treated with Phenol, o-Cresol, or with p-Nitrophenol—Electrophoretic analysis was carried out in two ways; either in the buffered solution of these agents as previously described or in the Miller-Golder's buffer solution. In the latter case, the RNP solutions were treated overnight with the agents, centrifuged in the cold, and then the supernatants were dialysed against the cold Miller-Golder's buffer solution repeatedly changing it so as to eliminate them completely from the samples.

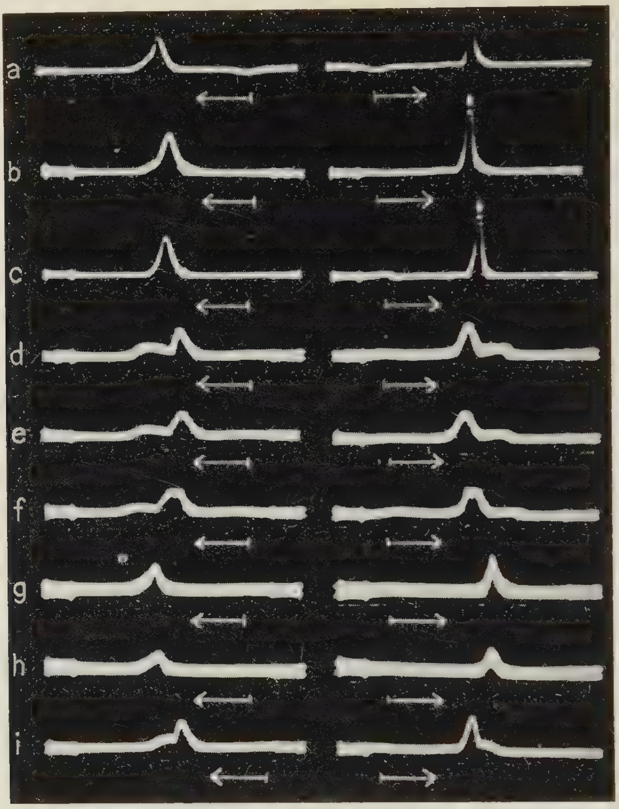

FIG. 1. Electrophoretic patterns of the microsomal RNP treated with phenol (a, d, g), *o*-cresol (b, e, h) and *p*-nitrophenol (c, f, i).

	Treatment	Solvent for electrophoresis
a :	3 per cent phenol	buffered 3 per cent phenol
b :	1.2 per cent *o*-cresol	buffered 2.4 per cent *o*-cresol
c :	0.5 per cent *p*-nitrophenol	buffered 0.5 per cent *p*-nitrophenol
d :	3 per cent phenol	Miller-Golder's buffer, pH 7.0, $\mu=0.1$
e :	1.2 per cent *o*-cresol	same above
f :	0.5 per cent *p*-nitrophenol	same above
g :	6 per cent phenol	same above
h :	2.4 per cent cresol	same above
i :	1.0 per cent *p*-nitrophenol	same above

Fig. 1-a, b and c illustrate the electrophoretic patterns of the RNP in 3 per cent phenol, 1.2 per cent *o*-cresol, and 0.5 per cent *p*-nitrophenol respectively. They are characterized by the remarkable homogeneity in an electric field, and their average ascending and descending mobilities in 10^{-5} cm^2. per sec. per volt were -11.5 and -10.8 (phenol), -12.2 and -11.6 (*o*-cresol), -12.3 and -11.8 (*p*-nitrophenol) respectively. These values are larger than the electrophoretic mobilities of the RNP (*8*), but are considerably smaller than these of free yeast RNA.

Fig. 1-d, e and f illustrate the electrophoretic patterns of the RNP prepared by the latter method after treatment with 3 per cent phenol, 1.2 per cent *o*-cresol or with 0.5 per cent *p*-nitrophenol respectively. It is observed that the homogeneous peaks of Fig. 1-a, b, and c were dissociated to the two peaks of a small faster and a large slower one probably by the autodegradation during dialysis. The average ascending and descending mobilities of the former were -15.8 and -15.1×10^{-5} cm^2. per sec. per volt which were equal to those of free RNA.

Fig. 1-g, h, and i also illustrate the electrophoretic patterns of the RNP analysed by the latter method after treatment with the buffer solution saturated with phenol (6.0 per cent) *o*-cresol (2.4 per cent), or with *p*-nitrophenol (1.0 per cent) respectively. The former two were electrophoretically homogeneous and their average and descending mobilities in the same unit were -13.9 and -13.8 (phenol), -14.1 and -13.6 (cresol) respectively. Similar electrophoretic patterns were obtained when the RNP was treated with the buffer solution saturated with phenol vigorously stirring them for 60 minutes at room temperature as Kirby described (*1*).

After electrophoresis, the samples were pipetted out of the cell, and the amount of RNA and protein were determined to compare them with those of the original RNP. Table II shows their RNA content and the recovery of RNA to the supernatants by the centrifugation at $2{,}500\times g$ for 15 minutes after treatment with phenol, *o*-cresol, or *p*-nitrophenol.

TABLE II

Recovery of RNA to Supernatants after Treatment of the RNP with Either Phenol, o-Cresol, or p-Nitrophenol and Their RNA Content

	Phenol		*o*-Cresol		*p*-Nitrophenol	
	3% (*w*/*v*)	6% (*w*/*v*)	1.2% (*w*/*v*)	2.4% (*w*/*v*)	0.5% (*w*/*v*)	1.0% (*w*/*v*)
Recovery of RNA to supernatant	93.5 %	58.4 %	87.9 %	49.1 %	79.6 %	84.9 %
RNA/RNA-protein of supernatant	52.1 %	67.8 %	49.2 %	64.8 %	43.1 %	50.2 %

It is shown that the RNPs treated with 3 per cent phenol, 1.2 per cent *o*-cresol, or 0.5 per cent *p*-nitrophenol contain considerable amount of protein, their RNA content being only about 50 per cent. Their remarkable homogeneity in an electric field, therefore, shows that RNA and protein do not exist independently but exist as a RNA-protein complex. These agents probably precipitated the degraded RNP with less RNA content such as Component 3 (*8*) selectively rather than disrupted the hydrogen bonds between RNA and protein of the RNP. Six per cent phenol or 2.4 per cent *o*-cresol, seems to have more effectively isolated RNA from the RNP. The isolated RNA, however, contained a considerable amount of protein as well. Such coexistence of protein with extracted RNA was also observed when the RNP solution was treated with water saturated phenol or *o*-cresol for 60 minutes at room temperature according to the Kirby's method (*1*), RNA content of the extract being only about 70 per cent.

Centrifugal Method—Two kind of RNP were used for this experiment. The one is the purified RNP prepared by the method described previously, and the other is the loosely packed sediments obtained by the centrifugation as 20,000×*g* of the resuspended deoxycholate insoluble fraction.

In the former case, 9 ml. of cold phenol or *o*-cresol solution was added to 1 ml. of the RNP solution (concentration 1.3 mg./ml.) to obtain 4/4, 3/4, 2/4 and 1/4 saturated solution respectively (saturated phenol solution was about 6.0 g./dl. and that of *o*-cresol about 2.4 g./dl. in the cold). Standing still overnight, these solution were centrifuged at 105,000×*g* for 120 minutes in the cold. RNA and protein either in the sediments and in the supernatants were determined by the method previously described.

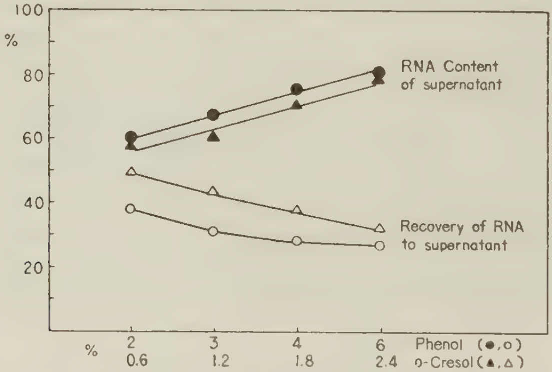

FIG. 2. Effect of phenol and *o*-cresol on the purified microsomal RNP studied by centrifugal method. The RNP treated with phenol or *o*-cresol overnight in the cold was centrifuged at 105,000×*g* for 120 minutes. RNA and protein either in the sediments and in the supernatants were determined and the recovery of RNA to supernatants and their RNA content were calculated.

Fig. 2 shows the recovery of RNA to the supernatants and their RNA content. It is noted that the RNA content is roughly proportional to the concentration of phenol or *o*-cresol, and yet the maximum RNA content was only about 80 per cent.

In the latter case, 1.5 ml. of cold phenol, *o*-cresol or *p*-nitrophenol was added to 0.5 ml. of the RNP solution of which concentration of RNA+protein was 10.5 mg./ml. to obtain 3/4 saturated, 2/4 saturated, and 1/4 saturated solution respectively (saturated *p*-nitrophenol was about 1.0 g./dl. in the cold).

Standing still overnight, in the cold, these solutions were centrifuged at 2,500×*g* for 15 minutes. RNA and protein either in the sediments and in the supernatants were also determined.

Fig. 3 represents one of the typical results, showing that the recovery of RNA to supernatant is satisfactory, while RNA content is always less than 70 per cent.

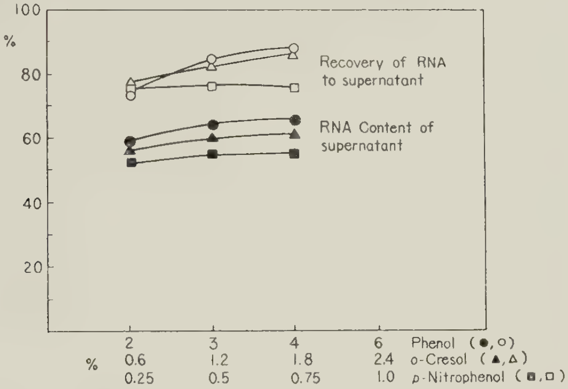

FIG. 3. Effect of phenol, *o*-cresol, and *p*-nitrophenol on the crude RNP studied by the centrifugal method. The crude RNP treated with phenol or *o*-cresol overnight in the cold was centrifuged at 2,500×*g* for 15 minutes and the recovery of RNA to supernatants and their RNA content were determined.

II. Divalent Phenols, Benzoic Acid and Its Derivatives

Effect of divalent phenols such as pyrocatechol, resorcine and hydroquinone on the RNP was examined by the similar methods as in the previous cases. Turbidity of the RNP solution hardly increased by the addition of pyrocatechol and resorcine, and so the RNP was analysed electrophoretically without centrifugation. As hydroquinone produced a slight turbidity increase after dialysis, RNP solution was centrifuged at 2,500×*g* for 15 minutes.

Fig. 4-c, d, e show the electrophoretic patterns of the RNP treated with pyrocatechol, resorcine and hydroquinone respectively. It is apparent that hardly any dissociation of the RNP was observed, though the electrophoretic patterns were considerably altered by these agents.

Effect of benzoic acid and its derivatives such as salicylic acid or *p*-aminobenzoic acid were examined as well, and it was found that the electrophoretic patterns of the RNP were also hardly affected by these compounds under our experimental conditions (Fig. 4-f, g, h).

III. Pyridine and α-picoline

Pyridine and picoline, which are known as effective proton acceptors, are reported to be able to dissolve actomyosin thread instantly, probably because of hydrogen-bond disruption (*14*).

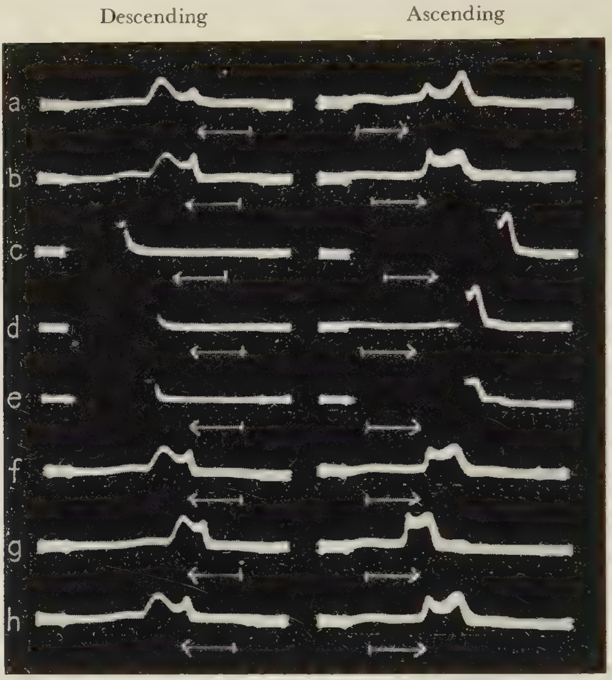

FIG. 4. Electrophoretic pattern of the microsomal RNP treated with various hydrogen bond forming agents. (a) 0.5 per cent 2,4-dinitrophenol, (b) 0.5 per cent *m*-aminophenol, (c) 2.5 per cent catechol, (d) 2.5 per cent resorcinol, (e) 2.5 per cent hydroquinone, (f) 0.2 per cent benzoic acid, (g) 0.2 per cent *p*-amino benzoic acid, (h) 0.2 per cent salicylic acid. Electrophoresis was carried out in the corresponding buffered solution of these agents.

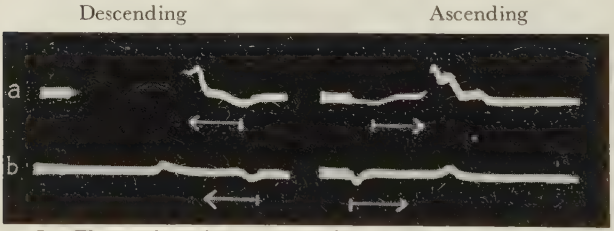

FIG. 5. Electrophoretic patterns of the RNP treated with 5 per cent (a) and 10 per cent (b). Electrophoresis was carried out in the corresponding buffered solution of pyridine. In the latter case, as the turbidity of the RNP solution remarkably increased, the sample was electrophoretically analysed. In these patterns it is noted that δ and ε boundaries are reversed.

Paperchromatographic Analysis—When the RNP was analysed paperchromatographically in 25 per cent pyridine or in α-picoline, a considerable amount of RNA were found near solvent front, while all the protein are detected at the starting line. In 50, 75 and 100 per cent (*v/v*) pyridine or *α*-picoline neither RNA nor protein migrate at all. These findings suggest the possibility that the RNP might be dissociated in dilute pyridine or in *α*-picoline.

Electrophoretic analysis—Pyridine was added to the RNP solution at a concentration of 5, 10, 20, or 40 per cent (*v/v*) respectively, and these solutions were dialysed overnight against the corresponding buffered solution of pyridine. In the latter three cases, turbidity of the RNP solutions gradually increased, hence they were centrifuged after dialysis at 2,500×*g* 15 minutes, and the supernatants were analysed electrophoretically.

Fig. 5-a is the electrophoretic patterns of the RNP treated with 5 per cent pyridine, where the peak designated as Component 2 represents the RNP and Component 3 is probably a partially dissociated RNP (*8*). The mobility of the small faster peak (Component 1) was slightly less than that of free yeast RNA under the similar experimental conditions and probably represents the RNA released out of the RNP by pyridine. These patterns are, in some respects, similar to those of the RNP treated with 2.0 *M* urea (*7*). After centrifugation of the RNP treated with 10 or 20 per cent pyridine, electrophoretic analysis of the supernatant demonstrated only a small faster peak as show in Fig. 1-b, of which mobility was also slightly less than that of free yeast RNA under the similar experimental conditions. Chemical analysis of the sediments showed that they contained about two third as much RNA as the original RNP.

From these results, it may be concluded that the dissociation of the RNP by pyridine is incomplete and most of the bonds between RNA and protein of the RNP are not disrupted by pyridine. Picoline was somewhat less effective than pyridine.

DISCUSSION

The results of the present experiment are summarized in the Table I. Among various hydrogen bond forming compounds, phenol and *o*-cresol could effectively split the RNA-protein bonds. The other compounds such as *p*-nitrophenol, pyridine and *α*-picoline in addition to urea were somewhat effective. Such a difference probably depends on the strength of the hydrogen bonds between RNA and protein of the RNP relative to those between the added agents and the RNP and also on the concentration of the agents used (In this report, the agents which were hardly soluble in aqueous solution were used at or near their saturated concentration). In spite of such a difference it is probable that the RNA-protein bonds of the RNP contains hydrogen bonds.

It is to be noted, however, that the supernatants of the RNP obtained by centrifugation after treatment with phenol or *o*-cresol contained a considerable amount of protein. As these agents have been known as protein coagulant, and their electrophoretic homogeneity was satisfactory, this protein is not free one but probably bound with RNA as a RNA-protein complex. It is estimated, therefore, that the microsomal RNP contains bonds other than hydrogen bonds. Some of them are RNA-amino acid complexes described by Potter and Dounce (*15*) or by Hoagland *et al.* (*16*), however the previous report that the RNP is dissociated to the two moieties in strong saline (*3*) suggests that ionic bonds are also important for the maintenance of the RNA-protein bindings.

Certain relevant conclusions may be drawn by examining the methods used for the preparation of RNA. For example, extraction with dilute alkali (*17*), 10 per cent sodium chloride (*18*) and detergents such as sodium dodecyl sulfate (*19*) seems to be related principally with the splitting of ionic bonds, while Kirky's method (*1*) is probably related with the disruption of hydrogen bonds.

As the bonds which are considered to be responsible for the binding of RNA to protein are usually also responsible for keeping protein molecules in their native state, it is ambiguous whether these agents are primarily effective in splitting the RNA-protein bonds or the isolation of RNA is only a secondary effect of denaturation of protein moiety. For example, sodium dodecyl sulfate have been known as a strong protein denaturant, and the Kirby's method (*1*) probably accompanies not only protein denaturation by phenol itself but also surface denaturation of protein at the phenol-water interface.

Such a consideration seems very important as long as the nature of bonds between RNA and protein is estimated from the conditions under which these bonds are disrupted, and it is probable too hasty to draw a definite conclusion as to the nature of the RNA-protein bond without further quantitative analysis on the molecular structure of the RNP and its constituents.

SUMMARY

Effect of various hydrogen bond forming agents such as phenol *o*-cresol, pyridine, α-picoline, *etc.* on the microsomal RNP were examined by electrophoretic and centrifugal method.

1. Phenol, *o*-cresol was found to be effective for the isolation of RNA from the RNP. Pyridine, α-picoline, *p*-nitrophenol, *etc.* were less effective than the former two. The other hydrogen bond-forming agents such as 2,4-dinitrophenol, *m*-aminophenol, pyrocatechol, resorcine, hydroquinone, benzoic acid, *p*-aminophenol, and salicylic acid were hardly effective under the present experimental conditions.

2. In spite of its homogeneity in an electric field, RNA isolated by centrifugation after the treatment of the RNP with phenol or *o*-cresol contained a considerable amount of protein.

3. It was suggested, therefore, that the RNP contains bonds other than hydrogen bonds and ionic bonds are also important for the RNA-protein bonds of the RNP particles.

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STUDIES ON THE AMINO ACIDS PRESENT IN YEAST RNA IN BOUND FORM*

I. LIBERATION OF AMINO ACIDS FROM RNA BY ACID HYDROLYSIS

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It has been reported in the previous communication (*1*) that the nucleic acids from several microorganisms and yeast which had been purified through the technique of paper electrophoresis liberated, upon acid hydrolysis, amino acids, the yield of which was found some 1 per cent on a dry weight basis with each sample material when expressed in term of glycine. Based on these observations a suggestion was given that these amino acids might have originated from peptides which were in combination with RNA with certain chemical bonds and inseparable in the electric field of electrophoresis applied.

The present paper deals with a study made in some detail on these amino acids, employing a variety of purified samples of yeast RNA. The data obtained show that the amino acids to be separable by acid hydrolysis might exist in association with RNA largely as single amino acid and in minor part as peptide.

EXPERIMENTAL AND RESULTS

Material—Use was made of various samples of yeast RNA of commercial products (Merck) as well as the RNA prepared from baker's yeast by the Crestfield method (*2*) in our laboratory. The purification procedure involved either a single performance of the chloroform-protein gel procedure by Sevag (*3*) or a combined use of the same procedure with continuous paper electrophoresis (*1*). Each of the purified RNA-Na (sodium salt of RNA) gave a negative test for the sensitive biuret reaction (*4*), behaved quite homogeneously in the electrophoresis apparatus by Tiselius both in a phosphate buffer at pH 6.8 and in a borate buffer at pH 8.0 and showed its absorption maximum in the ultraviolet at 262 mμ and its minimum at 232 mμ. The analytical results obtained are presented in Table I.

Qualitative Determination of Amino Acids Liberated upon Acid Hydrolysis of the Purified Yeast RNA

Acid Hydrolysis—15 mg. of each of the samples 1–3 were hydrolyzed with

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1.0 ml. of 6 N HCl at 100° for 20 hours and the hydrolysates, after treatment with ion exchangers Dowex-50 and Dowex-1, were analyzed for amino acids by two-dimensional paper chromatography in two solvent systems, (I) phenol-water (80 : 20), and (II) butanol-acetic acid-water (4 : 1 : 5), revealing 6 to 7 amino acids as indicated in Table II.

TABLE I

Yield and Analytical Data of the Purified Yeast RNA-Na

Sample No.	Yield involved in purification (%)	N (%)	P (%)	N/P	Atomic N : P	Total amino acids expressed in term of glycine (%)[2]
1	—	14.36	7.76	1.85	4.10	1.77
1-a	70	13.97	8.06	1.73	3.83	1.05
1-b	57	13.65	8.18	1.67	3.70	0.87
2	72	14.02	7.94	1.76	3.90	1.14
3	0.36[1]	13.40	7.64	1.75	3.88	1.05

Sample 1 : untreated commercial RNA-Na specimen (Merck).
,, 1-a : sample 1 was treated by the Sevag method.
,, 1-b : sample 1-a was subjected further to continuous paper electrophoresis (*1*).
,, 2 : another commercial RNA-Na preparation differing from sample 1 in vial number was purified by the Sevag method.
,, 3 : this RNA-Na was prepared from baker's yeast by Crestfield method and deproteinized by the Sevag procedure.

1) The yield from a pressed yeast cake.
2) 6 N HCl, 100°, 20 hour hydrolysis.

TABLE II

Amino Acids Found in Hydrolysates of the Purified Yeast RNA-Na

Sample	Number of amino acids found	Kind of amino acids		
		Acidic	Neutral	Basic
1-a	6	Asp, Glu	Ser, Gly, Val	His
1-b	6	Asp, Glu	Ser, Gly, Val	His
2	7	Asp, Glu	Ser, Gly, Val, Phe	His
3	7	Asp, Glu	Ser, Gly, Val, Phe	His

Alkaline Hydrolysis—Each 300 mg. portion of the samples 1–3 was hydrolyzed with 4 N KOH at 100° for 10 hours. The hydrolysates, after treatment with ion exchangers as mentioned above, were analyzed for amino acids by the use of two-dimensional paper chromatography as well as paper electrophoresis. The amino acids were identical with those found in acid hydrolysates. Attempt was made in this case to detect tryptophan by the ninhydrin as well as the Ehrlich reaction (*5*), but with negative results.

Quantitative Analysis for the Amino Acids Formed upon Acid Hydrolysis of the Purified Yeast RNA-Na—The two purified samples of 1-a and 1-b were analyzed for this purpose. Each 45 mg. portion of the samples was hydrolyzed with 2.0 ml. of redistilled 6 *N* HCl in a sealed tube at 100° for 20 hours. After removal of excess HCl by three times repeated evaporation with water in a vacuum desiccator, the residue was dissolved in 0.5 ml. of a citrate buffer at pH 3.0 and chromatographed on a 0.9×100 cm. column of Dowex-50

TABLE III

The Composition of the Amino Acids Found in Hydrolysates of the Purified Yeast RNA-Na (Sample 1-a)

20 hour hydrolysis of 45 mg. sample with 6 *N* HCl at 100°.

Amino acid	Area1)	Area/μM L-valine area	Conversion factor2)	Amount in			Molar ratio3)
				μM	μg	%	
Unknown	0.06	—	—	—	—	—	—
Asp	0.61	0.59	0.98	0.60	78	0.173	1.50
Ser	0.45	0.43	0.96	0.45	47	0.105	1.12
Glu	0.94	0.90	0.97	0.93	137	0.304	2.32
Gly	3.72	3.58	1.03	3.48	261	0.580	8.70
Val	0.42	0.40	1.00	0.40	47	0.104	1.00
His	0.32	0.31	0.74	0.42	65	0.145	1.05
Total				6.28	635	1.41	

1) Planimeter reading.

2) The color yield per μmole of individual amino acids expressed relative to the valine value as 1.00.

3) Values calculated on a molar basis when the value for valine was taken 1.00 as the standard.

TABLE IV

The Composition of the Amino Acids Found in Hydrolysates of the Purified Yeast RNA-Na (Sample 1-b)

20 hour hydrolysis of 45 mg. sample with 6 *N* HCl at 100°.

Amino acid	Area	Area/μM L-valine area	Conversion factor	Amount in			Molar ratio
				μM	μg	%	
Unknown	0.05	—	—	—	—	—	—
Asp	0.24	0.23	0.98	0.23	31	0.069	0.63
Ser	0.32	0.30	0.96	0.32	34	0.075	0.83
Glu	0.38	0.36	0.97	0.38	56	0.124	0.98
Gly	3.69	3.55	1.03	3.45	259	0.575	8.95
Val	0.40	0.38	1.00	0.38	45	0.100	1.00
His	0.30	0.28	0.74	0.39	60	0.135	1.01
Total				5.15	485	1.08	

(200–500 mesh) in sodium form following the method by Moore and Stein (*6, 7*) with slight modification (*8*). The data thus obtained are shown in Table III and IV.

Glycine Formation by the Action of HCl on Nuclein Bases—As shown in Table III and IV glycine is characterized by its exceedingly high value in the amino acid composition of the hydrolysates of the purified yeast RNA. It seemed, therefore, necessary to examine the nuclein bases which are responsible for the glycine formation. For this purpose the individual purine and pyrimidine bases were heated with 6 *N* HCl at 100° for 20 hours respectively, and the resulting solution, after removal of HCl, was dissolved in 0.5 ml. of a citrate buffer at pH 3.0 and analyzed for glycine content according to Moore and Stein, the results of which are given in Table V.

TABLE V

Glycine Formation by the Action of HCl on Nuclein Bases
(6 *N*, 100°, 20 hours.)

Sample	% yield of glycine on a dry weight basis of material
Cytosine	0.0
Uracil	0.0
Guanine	0.2
Adenine	8.2
3- adenylic acid	3.4 (or 8.5 regarding to adenine)
RNA sample 1-a	0.58
,, 1-b	0.58
,, 2	0.60

TABLE VI

Glycine Formation by the Action of HCl on Adenine under Varying Conditions
Glycine was estimated by the ninhydrin photometric method.

Concentration of HCl	% yield of glycine from adenine by heating at 100° for				
	1 hr.	3 hr.	6 hr.	12 hr.	20 hr.
1 *N*	0.0	0.2	0.8	1.3	3.7
3 *N*	0.5	0.8	2.2	4.4	7.7
6 *N*	0.5	0.9	2.6	5.0	8.5

It has been established by the preceding experiments that adenine plays among other bases a principal role in forming glycine when RNA is hydrolyzed with conc. HCl. Consequently, a study was made to see under what conditions and to what extent adenine can give rise to glycine. The result is summarized in Table VI.

From Table VI it is obvious that the glycine formation from adenine tends to increase as the concentration of HCl is raised or the duration of heating is prolonged.

Actual Yield of Amino Acids by Acid Hydrolysis of RNA with Due Consideration for Glycine Originating from Adenine—RNA was hydrolyzed with 1 *N*, 3 *N*, and 6 *N* HCl at 100° during various periods of time and the total amino acids liberated in the hydrolysates were estimated spectrophotometrically employing glycine as the standard. On the other hand the same amount of RNA was hydrolyzed under identical conditions and the glycine formation was traced in this case by the method of Moore and Stein. The whole data are presented in Fig. 1.

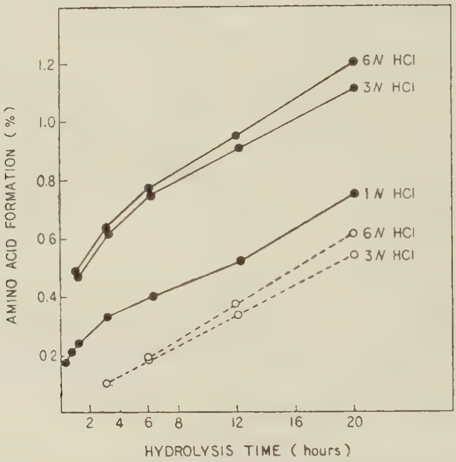

FIG. 1. Total amino acids formed by acid hydrolysis of yeast RNA in comparison with the glycine formation from the RNA under identical conditions. ---○--- actual glycine formed by hydrolysis with 3 *N*, and 6 *N* HCl at 100°. —●— total amino acids formed by hydrolysis with 1 *N*, 3 *N*, and 6 *N* HCl at 100°, the amounts being expressed in term of glycine.

Fig. 1 demonstrates that the yield of the total amino acids from RNA increases with the lapse of the hydrolysis time rather rapidly during the first period of about 6 hours, after which the liberation significantly declines until it proceeds at a slower but constant rate. The glycine production from RNA, on the other hand, is brought about at a constant rate from the initial stage of hydrolysis. It is noticeable to find that an appreciably parallel corelation seems to exist between the production curves of the total amino acids from RNA and those of glycine from RNA, especially in the region following 6 hour hydrolysis. From this comparison, it is probable that the RNA bound amino acids can be separated almost entirely by hydrolysis with either 3 *N* or 6 *N* HCl at 100° for 6 hours and that the increased production of the total amino acids which takes place afterwards by prolonged

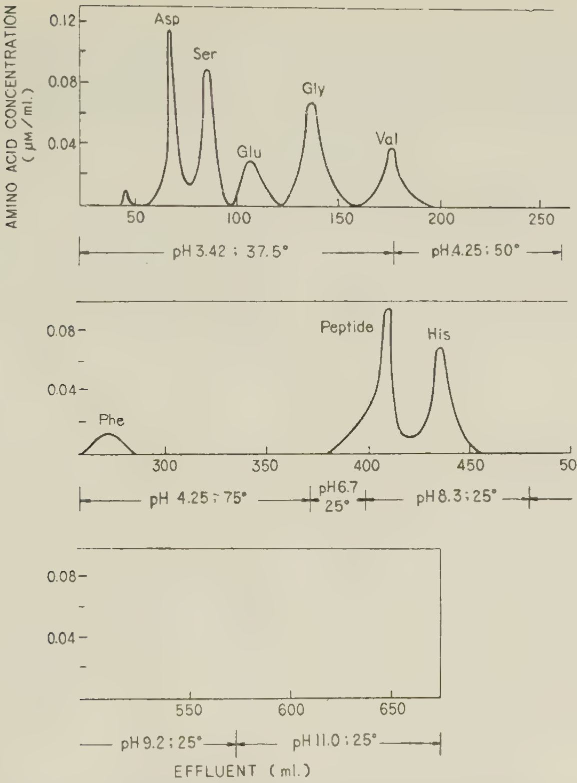

FIG. 2. Analysis by column chromatography for amino acids bound to the purified yeast RNA-Na sample 2. 3 hour hydrolysis of 50 mg. sample with 3 N HCl at 100°.

TABLE VII

The Composition of the Amino Acids Found in Hydrolysates of the Purified Yeast RNA-Na (Sample 2)

3 hour hydrolysis of 50 mg. sample with 3 N HCl at 100°.

Amino acid	Area	Area/μM L-valine area	Conversion factor	Amount in			Molar ratio
				μM	μg.	%	
Unknown	0.05	—	—	—	—	—	—
Asp	0.52	0.50	0.98	0.51	66	0.132	1.76
Ser	0.50	0.43	0.96	0.45	47	0.094	1.55
Glu	0.22	0.21	0.97	0.22	32	0.064	0.76
Gly	0.66	0.63	1.03	0.61	46	0.092	2.10
Val	0.30	0.29	1.00	0.29	34	0.068	1.00
Phe	0.18	0.17	0.93	0.18	30	0.060	0.62
Peptide	0.66	0.64	—	—	—	—	—
His	0.64	0.62	0.74	0.84	130	0.260	2.90
Total				3.10	385	0.77	

hydrolysis is due to the additional formation of glycine from adenine. With adequate consideration for these hydrolysis conditions two specimens of the purified RNA sample 2 were hydrolyzed with 3 *N* HCl at 100°, one specimen for 3 hours and the other for 6 hours. Fig. 2 and 3 illustrate the chromatographic patterns of the amino acids thus obtained and their numerical data are summarized in Table VII and VIII.

As can be seen in Fig. 2 and Table VII, in case of 3 hour hydrolysis a peptide fraction appears just before the histidine peak. This peptide, however, disappears in case of 6 hour hydrolysis with simultaneous augumentation of the other amino acids as presented in Fig. 3, and Table VIII. From this it may be said that an entire separation of amino acids from RNA is achieved by heating with 3 *N* HCl at 100° for 6 hours. Thus, the RNA bound amino acids can be represented by those amino acids which are liberated by 3 *N* HCl-100°-6 hour hydrolysis minus glycine which is formed from adenine under identical conditions.

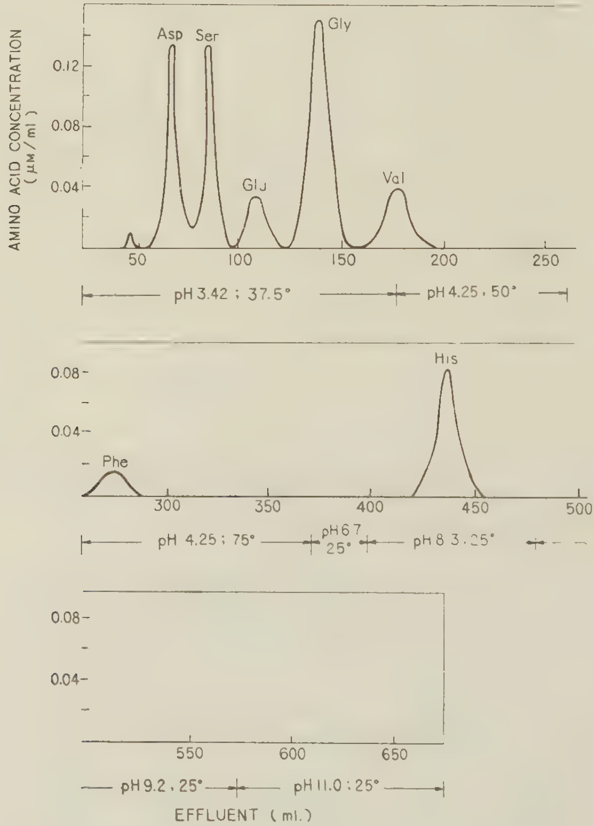

FIG. 3. Analysis by column chromatography for amino acids bound to the purified yeast RNA-Na sample 2. 6 hour hydrolysis of 50 mg. sample with 3 *N* HCl at 100°.

Amino Acid Liberation by Alkaline Hydrolysis of Yeast RNA—Hydrolysis of RNA with 1 *N* KOH separated amino acids faster than with 1 *N* HCl, in other words, at a rate of about twice as high as with acid. In case of heating with 1 *N* KOH at 100° for 1 hour the liberation of amino acids approached 80 per cent of the yield to be attained by hydrolysis with 4 *N* KOH at 100° for 12 hours. In addition, it should be mentioned that none of the nuclein bases other than adenine provides practically any glycine by treatment with alkali or acid. The whole data are listed up in Table IX.

TABLE VIII

The Composition of the Amino Acids Found in Hydrolysates of the Purified Yeast RNA-Na (Sample 2)

6 hour hydrolysis of 50 mg. sample with 3 *N* HCl at 100°.

Amino acid	Area	Area/μM L-valine area	Conversion factor	Amount in μM	Amount in μg.	Amount in %	Molar ratio
Unknown	0.06	—	—	—	—	—	—
Asp	0.64	0.62	0.98	0.64	84	0.167	1.88
Ser	0.64	0.62	0.96	0.64	68	0.137	1.88
Glu	0.28	0.26	0.97	0.26	38	0.077	0.76
Gly	1.28	1.24	1.03	1.20	90	0.180	3.52
Val	0.36	0.34	1.00	0.34	40	0.081	1.00
Phe	0.20	0.20	0.93	0.20	34	0.068	0.61
His	0.72	0.70	0.74	0.94	146	0.292	2.47
Total				4.22	500	1.00	

TABLE IX

Test for Amino Acid Liberation from Yeast RNA and Nuclein Bases by the Action of Acid or Alkali

Hydrolysis at 100° Agent	Hydrolysis at 100° Time	% yield of amino acids in term of glycine from RNA	Uracil	Cytosine	Adenine	Guanine
1 *N* HCl	10 mins.	0.17	0.0	0.0	0.0	0.0
	30 ,,	0.20	0.0	0.0	0.0	0.0
	60 ,,	0.24	0.0	0.0	0.0	0.0
6 *N* HCl	20 hrs.	1.19	0.0	0.0	8.5	0.2
1 *N* KOH	10 mins.	0.34	0.0	0.0	0.0	0.0
	30 ,,	0.43	0.0	0.0	0.0	0.0
	60 ,,	0.52	0.1	0.1	0.0	0.0
4 *N* KOH	12 hrs.	0.64	—	—	—	—

DISCUSSION

It is very likely that the amino acids liberated by acid hydrolysis from the purified yeast RNA is in major part linked with the nucleic acid molecule as a single amino acid and in minor part as peptides. In support for this assumption is the fact that the individual amino acids are liable to be split off almost entirely by 6 hour hydrolysis with 3 *N* HCl at 100° and even by 1 hour hydrolysis with 1 *N* HCl at 100° to an extent of 30 per cent, although there was observed simultaneous production of glycine from adenine which was, therefore, to be checked for yield evaluation of actually bound amino acids. According to the author's experience, tripeptides, *e.g.*, glycyl-leucyl-glycine and alanyl-leucyl-glycine suffer no significant decomposition by heating with 1 *N* HCl at 100° for 1 hour, but an incomplete hydrolysis with 6*N* HCl at 100° for 5 to 10 hours and a complete hydrolysis with 6 *N* HCl at 100° for 15 hours. As indicated in Fig. 2, and Table VII, an eluting fraction containing peptide was obtained in a yield of some 20 per cent of the total amino acids on a ninhydrin color basis, however, the chemical characteristics of the peptide still remains uncertain.

In so far as the chemical bond between the amino acids and the nucleic acid moiety is concerned, the most probable form may be an ester linkage. In favor of this view is the observation that the bound amino acid is more susceptible to alkali than to acid, as shown in Table IX. A recent study made by Lipmann *et al.* (*9*) on soluble rat liver RNA employing radioactive carbon as a marker evidenced that the amino acid and the RNA are joined together with an ester bond formed between amino acid and ribose, *i.e.*, an amino acyl ribose type and not a type of amino acid-phosphoryl anhydride. This is based on the fact that C^{14}-leucyladenosine was obtained upon paper electrophoresis of the hydrolysate of C^{14}-leucine incorporated RNA. In one of our recent studies on alkaligenic acid, a special type of nucleic acid containing an appreciable amount of polypeptide-sugar complex in bound form (*10*), it was reported that partial hydrolysis of this compound gave rise to a ribose bound polypeptide which was detected chromatographically, indicating a possible existence of peptides esterified with ribose molecule. This observation may be in accord with the finding in the present work on the point that amino acids can be esterified with the ribose residue.

Some amino acids such as glutamic acid, aspartic acid and serine appear to be present associated with RNA to certain extent in a form of ionic linkage. This may be evident when comparison is made between Table III and IV on the values for those amino acids. The yeast RNA 1-a, *i.e.*, a sample deproteinized by the Sevag procedure, contain more glutamic acid (2.5 times), aspartic acid (2.5 times), and serine (1.4 times) than the yeast RNA 1-b, *i.e.*, a further purified RNA (1-a) sample by continuous paper electrophoresis. This may mean the existence of loosely bound amino acids which can be released from the linking with RNA in an electric field of certain strength applied.

Concerning the small peak of the chromatographic elution curve which appears in the region of the 46th collector tube, no information is available about its chemical characteristics as yet. A similar peak has already been observed in the studies on alkaligenic acid (*10*) and also with an ox-spleen DNA sample purified by Sevag.* Both of urea and taurine which appear close to the same region were rejected by chemical tests made with the corresponding effluents.

As regards the glycine formation from purine by the action of HCl, Kossel (*11*) in an earlier report observed that adenine was not affected by heating with conc. HCl at 100°, but it suffered degradation at higher temperature, yielding CO_2 and NH_3. Krüger (*12*) stated that adenine could be heated with conc. HCl (s.g. 1.19) at 180–200° for 12–14 hours or at 135° for a longer period of time, being accompanied by its decomposition into NH_3, CO_2, glycine, and formic acid. According to the more recent work by Frick (*13*) adenine decomposed by heating with 0.9 *N* HCl at 100° for 2 hours with simultaneous liberation of glycine. Anyway, careful consideration in a quantitative way of the glycine formation from a nuclein base adenine is of prime importance in the study of amino acids combined with RNA.

SUMMARY

1. Various purified samples of yeast RNA from several origins yielded upon acid hydrolysis about 1 per cent of amino acids of 6 to 7 varieties.

2. These amino acids consisting of acidic, neutral, and basic ones seem to exist linking with RNA mostly as a single amino acid rather than as peptide.

3. A sample deproteinized by Sevag differs from a sample obtained by further purification by continuous paper electrophoresis in its amino acid composition in such a way that glutamic acid, aspartic acid, and serine are appreciably reduced in the latter sample.

4. Lability of the bound amino acids to alkali was found greater than to acid.

5. The actually bound amino acids can be obtained by hydrolysis of the purified sample with 3 *N* HCl at 100° for 6 hours and by checking the observed total amino acids for the glycine originating from decomposed adenine.

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ON THE PREPARATION OF ADENOSINE TRIPHOSPHATE-γ-P^{32}* AND ADENOSINE DIPHOSPHATE-β-P^{32}

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ATP^{32}** in which the three phosphorus atoms are labeled with P^{32} with different specific radioactivity is required to clarify the mechanism of transpyrophosphorylation. Although several enzymological and chemical methods for preparation of the ATP^{32} have been reported, almost all of them lacked detailed data of the analysis of the preparation. In order to prepare ATP-γ-P^{32}, Kornberg *et al.* (*1*) described a method of utilizing acetokinase to transfer P^{32} to ADP from acetyl phosphate-P^{32}, which was synthesised by acetylation of iP^{32} with acetic anhydride. Wieland and Pfleiderer (*2*) and Gleiling (*3*) reported the synthesis of ATP-γ-P^{32} by the use of triose phosphate dehydrogenase, phosphoglycerate kinase, iP^{32}, ADP and DPN. While Lowenstein (*4*) tried the chemical synthesis of ATP-γ-P^{32} with iP^{32}, ADP and DCC, phosphorus atoms at the β- and γ-positions had no markedly different radioactivity in his product. An enzymic preparation of ATP-β-P^{32} was reported by Kornberg *et al.* (*1*), in which ATP-β,γ-P^{32} previously prepared by rat liver mitochondria was treated with hexokinase and glucose to remove its terminal P^{32}, and the resulting ADP-β-P^{32} was phosphorylated to ATP-β-P^{32} by the action of pyruvate kinase with phosphoenolpyruvate.

Of several methods (*4–6*) for analyzing the specific activity of each phosphorus atom in the ATP^{32} molecule, the method of Kornberg and Pricer (*5*) is the most interesting. According to their method using hexokinase, adenylate kinase and glucose, a sample of ATP^{32} was converted to AMP and glucose-6-phosphate, which were separated and counted to evaluate the radioactivity of α-P and that of β- and γ-P. Utilizing the potato apyrase which detaches γ-P alone or both β-P and γ-P depending on the reaction temperature when splitting iP from ATP, Lowenstein (*4*) determined the specific activity of each phosphorus atom by measuring iP^{32} liberated from ATP^{32} by the apyrase after extraction of iP^{32} with isobutanol.

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* The three phosphorus atoms of adenosine triphosphate are designated as α, β and γ, thus: adenosine-P^{α}-P^{β}-P^{γ}.

** Abbreviations used: ATP or AR-P-P-P, adenosine-5-triphosphate; ADP or AR-P-P, adenosine-5-diphosphate; AMP or AR-P, adenosine-5-monophosphate; iP, inorganic orthophosphate; CAP, carbamyl phosphate; DCC, *NN'*-dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane.

The present report describes 1) preparation of ATP-γ-P^{32} and ATP-β-P^{32}, which have high specific activities and marked differences between the radioactivity of each phosphorus atom sufficient for practical use, and 2) convenient methods for analysis of ATP^{32} relative to P^{32}.

EXPERIMENTAL

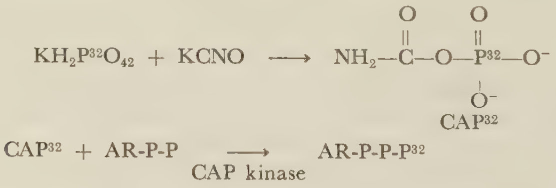
I. Methods for Separation of AMP, ADP, ATP and iP

(*i*) *Column Chromatography*—Dowex-1 (× 4 per cent)-Cl^-, 200–400 mesh, was used as an exchanger. Nucleotides were eluted successively with NaCl-HCl eluting reagent system by the method of Cohn and Carter (*7*), and then evaluated by extinction at 260 mμ or 257 mμ with a spectrophotometer (Hitachi Ltd.). Radioactivities were determined with a Geiger-Müller counter (The Institute of Physical and Chemical Research) after aliquots of each fraction were evaporated to dryness on counting discs.

(*ii*) *Paper Chromatography*—Paper chromatography was carried out with Toyo filter paper No. 53. A solvent system of butyric acid, 1 *N* ammonium hydroxide and 0.1 *M* EDTA (60 : 36 : 1, by volume), which is a slight modification of the solvent used by Krebs and Hems (*8*), was mainly used for descending chromatography. Spots of nucleotide components on the chromatograms were detected by their absorption of ultraviolet light in the region of 260 mμ according to the procedure of Markham and Smith (*9*). Radioautograms of the chromatograms were prepared with Fuji X-ray films.

II. Preparation of ATP-γ-P^{32}

The preparation of ATP-γ-P^{32} made use of a phosphate transferring reaction from carbamyl phosphate to ADP discovered by Jones *et al.* (*10*). It consisted of chemical and enzymic steps as follows:



$$KH_2P^{32}O_{42} + KCNO \longrightarrow \underset{CAP^{32}}{NH_2{-}\overset{\overset{\Large O}{\|}}{C}{-}O{-}\underset{\underset{\Large O^-}{|}}{\overset{\overset{\Large O}{\|}}{P^{32}}}{-}O^-}}$$

$$CAP^{32} + AR\text{-}P\text{-}P \xrightarrow[\text{CAP kinase}]{} AR\text{-}P\text{-}P\text{-}P^{32}$$

Because CAP^{32} can be easily synthesized in a high yield in terms of P^{32} by the first reaction and the equilibrium of the second reaction is greatly in favor of ATP formation as reported by Jones *et al.* (*10*), these reactions were used to synthesize ATP-γ-P^{32}.

(*1*) *Chemical Step*

CAP was prepared by a slight modification of the method of Jones *et al.* (*11*). Following incubation of an aqueous solution of KH_2PO_4 and KCNO at 30°, $LiClO_4$ was added to the mixture to remove K^+ and residual phosphate and the CAP formed was precipitated by addition of ethanol. CAP was evaluated by the method of Spector *et al.* (*12*), determining iP liberated on heating at 100° for 2 minutes. At first, various conditions were examined in order to determine the best conditions to give the maximum yield of CAP in terms of P. Table I shows that the composition of reaction mixture and the reaction period in experiment II gave the best results in terms of yield of P, although the purity of the product was lower than that of the product obtained under experiment I. Therefore, the conditions of experiment II were selected as the experimental conditions for the following synthesis of CAP^{32}.

A solution of 0.4 mmole of KH_2PO_4 dissolved in dilute HCl solution containing 1 mc. of iP^{32} was evaporated to dryness and 0.4 ml. of water was added to the reaction vessel, followed by the addition of 0.8 mmole of KCNO. The reaction mixture was warmed at 30° for 60 minutes and then cooled in an ice bath. To the cold mixture, an ice-cold solution of 1.2 mmoles of LiOH, 0.134 ml. of 60 per cent $HClO_4$ (1.2 mmoles) and 0.4 ml. of water was added slowly. After removing the precipitate of $KClO_4$ and lithium phosphate by centrifugation, the Li salt of CAP^{32} was precipitated by addition of an approximately equal volume of ethanol. The precipitate was washed successively with ethanol and ether, and dried over phosphorus pentoxide in a vacuum desiccator.

TABLE I

Comparison of the Yields of CAP under Various Conditions

Experiment number	I	II	III	IV
KH_2PO_4 (*mmole*)	0.4	0.4	0.4	0.2
KCNO (*mmole*)	0.4	0.8	1.6	0.4
Water (*ml.*)	0.4	0.4	0.8	0.2
Incubation period (minute)	30	60	60	60
LiOH (*mmole*)	1.2	1.2	2.4	0.6
$HClO_4$ (*mmole*)	0.8	1.2	2.0	0.6
Water (*ml.*)	0.33	0.4	0.5	0.2
Yield in terms of P (%)	30	43	31	30
Purity (%)	57	53	48	—

Although the preparation contained $KClO_4$ and lithium phosphate as impurities, the ethanol fractionation adopted by Spector *et al.* (*12*) was omitted to recover Li-CAP^{32} completely, since some of the CAP was observed to be lost by this fractionation in previous experiments. Moreover, these impurities did not inhibit the enzymic reaction of ATP formation and were completely removed during the purification of ATP in the present method.

Evaluation by the method of Spector *et al.* (*12*) showed that CAP^{32}

formed was 0.17 mmol and the yield was 43 per cent in terms of P^{32}.

(2) *Enzymic Step*

(*i*) *Bacteria as the Enzyme Source*—The bacterial preparation containing carbamyl phosphate kinase was prepared from *Streptococcus faecalis* 8022 ATCC (supplied by the Institute for Infectious Deseases, University of Tokyo), which was cultured at 37° for 72 hours in a liquid medium containing human blood plasma and glucose. *S. faecalis* is usually cultured on a blood-agar medium, but when this medium is used, the bacteria may be contaminated with red blood cells which contain the enzymes randomizing the labeling of ATP^{32}. Therefore the blood-agar medium was not used in order to avoid such contamination. The yield of the organisms grown on a blood-agar slant was compared with that of the organisms cultured in the following media: an agar medium containing liver extract and whey, an agar medium containing bovine muscle infusion, a beef infusion broth, an agar medium containing human plasma and glucose, a liquid medium containing human plasma and glucose, and a liquid medium containing lyophilized human plasma and glucose. Because the liquid medium containing plasma and glucose gave the highest yield, this medium was selected for use. It was composed of 1.5 per cent NaCl, 0.1 per cent K_2HPO_4, 1.0 per cent tryptic digest of casein, 0.5 per cent glucose and *ca.* 7 per cent plasma. The cells, after harvest by centrifugation, were washed three times with physiological saline solution and once with distilled water, and then lyophilized. A satisfactory yield of cells (1.1 g. of fresh cells/1,250 ml.) was obtained after incubation at 37° for 72 hours. The lyophilized bacterial powder retained its enzyme activity for at least 3 months when stored in a desiccator in the cold.

(*ii*) *Enzyme Preparation*—A mixture of lyophilized powder of *S. faecalis* (0.6 g.) and glass powder (0.5 g.) was ground at 0° for 15 minutes with wetting amounts of Tris buffer, 0.1 *M*, pH 7.4. After stirring the gummy paste with 10 ml. of the buffer, the mixture was centrifuged at $18{,}000 \times g$ for 20 minutes in the cold. The precipitate was extracted again with 7 ml. of the buffer and centrifuged as before, and the clear supernatants were combined. Solid ammonium sulfate was added to the supernatant with stirring to obtain three fractions at 60, 70 and 80 per cent saturation (fractions a, b and c, respectively). Each of these three kinds of precipitated was dissolved in a small amount of Tris buffer and the activity of CAP kinase was checked.

In order to determine the activity of these fractions, the amount of CAP remaining unchanged was determined by the method of Spector *et al.* (*12*), after incubation of each fraction under the following conditions, in accordance with the method of Jones *et al.* (*11*). The reaction mixture containing 5 μmoles of Li-CAP, 5 μmoles of ADP, 10 μmoles of $MgCl_2$, 100 μmoles of Tris buffer, pH 7.4, and about 0.1 mg. of enzyme preparation in 0.9 ml. of final volume. The vessels were incubated at 30° for 20 minutes. The quantity of enzyme protein was checked by the comparison of optical density at 260 mμ with that at 280 mμ according to the method of Warburg and Christian (*13*).

It was observed in this experiment that the activity of CAP kinase in each fraction decreased in the order of fractions c, b and a, whereas the activity of destroying CAP and ADP diminished in the reverse order, *i.e.* fractions a, b and c. Consequently, fraction c was chosen for use as the enzyme preparation.

(*iii*) *Procedures for the Synthesis*—The incubation mixture for ATP^{32} synthesis consisted of 14 μmoles of Li-CAP^{32}, 80 μmoles of ADP, 80 μmoles of $MgCl_2$, 800 μmoles of Tris buffer, pH 7.4, and 1.5 mg. of enzyme preparation in total volume of 8.0 ml. It was incubated at 30° for 30 minutes. Addition of ADP in a relatively large amount was made not only to transfer iP^{32} fully from CAP^{32} to ADP, but also to diminish the randomization of the labeling pattern by the action of adenylate kinase which might contaminate the enzyme preparation. Following incubation, the reaction mixture was diluted ten-fold with water, adjusted to pH 8.0, and passed through a column of Dowex-1 (4.0×1.1 cm.) to allow adsorption of the reaction products, which were then eluted by the method of Cohn and Carter (*7*), as shown in Fig. 1.

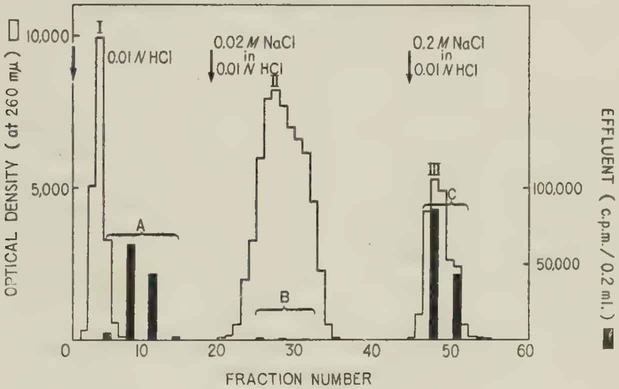


FIG. 1. Separation of the products formed during the synthesis of ATP-γ-P^{32}.
Exchanger: Dowex-1 (× 4 per cent)-Cl^-, 200–400 mesh, 4.0×1.1 cm. Eluting solution: HCl, NaCl as shown. Fraction volume: 10ml. Flow-rate: 2 ml./minute.

The first peak (I) in the ultraviolet absorption at 260 mμ corresponded to AMP, which was a mixture of AMP contained in ADP preparation as an impurity and AMP derived from ADP by disintegration during the reaction. The second (II) was remaining ADP, and the third (III) ATP formed. It was revealed from the amount of ATP^{32} as calculated from the last peak that almost all CAP^{32} was utilized for synthesis of ATP^{32}. The first peak (A) of radioactivity showed iP^{32}, the greater part of which was a contamination in the preparation of Li-CAP^{32}. It was expected from the low activity of the fraction of ADP (B) that randomization of labeling of ATP^{32} scarcely took place. The peak of ATP showed a high radioactivity (C).

The fraction of ATP^{32} was concentrated by passage through another small column of Dowex-1 (4.0×0.6 cm.), using cold 0.1 *N* HCl as eluting solution. From the ATP precipitated as Ba salt under the conditions of Le Page (*14*), Ba was removed by the use of Amberlite IR-120-H^{+}. Lyophilization of the solution of ATP after neutralization with NaOH solution afforded the Na salt of ATP^{32}.

(*iv*) *Results and Discussion*—Na-ATP^{32} obtained was 10 μmole. Yield was 72 per cent based on CAP^{32}, and *ca.* 30 per cent in terms of iP^{32}, the starting material. The ATP^{32} appeared as a single spot on paper chromatogram and two spots on radioautogram, one of which was intensive and the other very faint and small. The former corresponded to ATP and the latter to iP which might be derived from ATP disintegrated during chromatography. Specific activity of the ATP^{32} was 307,000 c.p.m. per μmole. The result given by the partial hydrolyzation analysis by acid treatment, which will be described later, showed that α-P was not labeled with P^{32} at all and that the ratio of specific activities of β-P to γ-P was 1:210. The slight random distribution of labeling was considered to be caused by the action of adenylate kinase which contaminated the enzyme preparation.

TABLE II

Results of the Preparation of ATP-γ-P^{32}

The incubation systems for the synthesis of CAP^{32} contained 400 μmoles (1 mc.) of $KH_2P^{32}O_4$, 800 μmoles of KCNO and 0.4 ml. of water (expt. I); 200 μmoles (4.5 mc.) of $KH_2P^{32}O_4$, 400 μmoles of KCNO and 0.2 ml. of water (expt. II); and 200 μmoles (6.1 mc.) of $KH_2P^{32}O_4$, 400 μmoles of KCNO and 0.2 ml. of water (expt. II); and 200 μmoles (6.1 mc.) of $KH_2P^{32}O_4$, 400 μmoles of KCNO and 0.2 ml. of water (expt. III). They were incubated at 30° for 60 minutes. After the addition of 1200 (expt. I) or 600 μmoles (expts. II and III) of $LiClO_4$ and centrifugation, CAP^{32} was precipitated with ethanol (see text).

The incubation mixtures for the preparation of ATP-γ-P^{32} consisted of 14 (expt. I), 23 (expt. II) and 60 μmoles (expt. III) of Li-CAP^{32}, 80 μmoles of ADP, 80 μmoles of $MgCl_2$, 800 μmoles of Tris buffer, pH 7.4, and 1.5 mg. of enzyme preparation in 8.0 ml. of final volume. The mixtures were incubated at 30° for 30 minutes.

Experiment number	iP^{32} used		CAP^{32} synthesized	CAP^{32} used	ATP^{32} obtained	Specific activity of ATP^{32}	Ratio of specific activity β-P/γ-P
	(*mc.*)	(*μmole*)	(*μmole*)	(*μmole*)	(*μmole*)	(×10^{3} *c.p.m./μmole*)	
I	1	400	170	14	10	307	1/210
II	4.5	200	—	23	17	2,655	1/ 32
III	6.1	200	—	60	44	3,857	1/ 11.5

Results of the preparation by the method mentioned above are shown in Table II. ADP-β-P^{32} was derived from ATP-γ-P^{32} by the action of adenylate kinase contained in the enzyme preparation. Increase in the amount of CAP^{32} resulted in augumentation of the ADP-β-P^{32}, namely the increase was considered to diminish the dilution of ADP^{32} by substrate ADP when other conditions were fixed. Therefore, the ratio of the specific activity of β-P to that of γ-P in the ATP^{32} prepared in experiment III, in which 60 μmoles of of CAP^{32} were used, was found to be larger than that in the ATP^{32} prepared in experiment I where 14 μmoles of CAP^{32} were used.

In this laboratory it was difficult to obtain a sufficient amount of *S. faecalis* to carry out further procedures for the purification of the enzyme, because of the lack of special equipment for culture of bacteria on a large scale. Diminishing the ratio of amounts of CAP to ADP was the most effective and simplest way to avoid the randomization of labeling by impurities in the enzyme preparation.

In the method of Kornberg *et al.* (*1*) with acetokinase, the yield in the step of acetylphosphate synthesis determines the overall yield. It was impossible by Kornberg's method, to obtain a yield of acetyl phosphate higher than 35 per cent based on iP, although efforts were made to increase the yield under various conditions. The method described in the present paper is thus regarded to be an efficient one which may be comparable to the procedure of Kornberg *et al.*

In addition when ATP-γ-P^{32} prepared by the present method was used to elucidate the mechanism of transpyrophosphorylation from ATP to thiamine by Shimazono *et al.* (*15*) in this laboratory, satisfactory results were obtained. This proved that the preparation of ATP-β-P^{32} had a high specific activity and a marked difference in activity between each phosphorus atom great enough for practical use, and a sufficient purity for enzymic experiment.

III. Preparation of ATP-β-P^{32}

This method also consisted of chemical and enzymic steps. The scheme of synthesis is as follows:

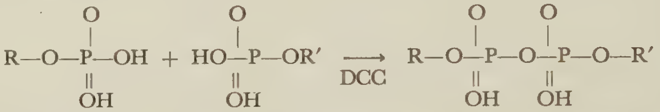
$$H_3P^{32}O_4 + \text{AR-P} \xrightarrow[\text{DCC}]{} \text{AR-P-P}^{32}$$

$$\text{AR-P-P}^{32} + \text{PEP} \xrightarrow[\text{Pyruvate kinase}]{} \text{AR-P-P}^{32}\text{-P}$$

No complicated procedures or special instruments are required in the method for synthesis of ADP^{32} with DCC, and moreover ADP^{32} of high specific radioactivity can be obtained with great ease. There is, however, a possibility that various by-products may be formed by this reaction. In case the method is applied to the synthesis of ATP-γ-P^{32} from ADP and iP^{32}, not only is it difficult to separate and purify the ATP^{32} but randomization of the labeling may also occur as reported by Lowenstein (*4*). In the present method for preparation of ATP-β-P^{32}, various nucleotides, which are synthesized in the chemical step along with ADP, are presumably excluded from the reaction process by the substrate specificity of the enzyme in the enzymic step. The equilibrium of the reaction favors the formation of ATP. For these reasons the enzymic reaction was chosen in the step for synthesis of ATP.

(*1*) *Chemical Step*

(*i*) *Tests on Reaction Conditions*—The reaction with DCC, in general, proceeds as follows:



$$\mathrm{R{-}O{-}\underset{\underset{\displaystyle OH}{\|}}{\overset{\overset{\displaystyle O}{|}}{P}}{-}OH + HO{-}\underset{\underset{\displaystyle OH}{\|}}{\overset{\overset{\displaystyle O}{|}}{P}}{-}OR' \xrightarrow[DCC]{} R{-}O{-}\underset{\underset{\displaystyle OH}{\|}}{\overset{\overset{\displaystyle O}{|}}{P}}{-}O{-}\underset{\underset{\displaystyle OH}{\|}}{\overset{\overset{\displaystyle O}{|}}{P}}{-}O{-}R'}$$

When AMP and iP are used as reactants, there is a possibility that many kinds of nucleotides and polyphosphates are produced, depending on the combination of two phosphorus compounds. Accordingly, the condition for synthesis of ADP^{32} was investigated in order to obtain the highest yield based on P^{32} by varying the ratio of the amounts of AMP to iP^{32} and the reaction time. The procedures followed principally the method of Lowenstein (*4*). After addition of a certain amount of H_3PO_4 as a carrier to a solution containing iP^{32} dissolved in dilute HCl, the mixture was evaporated to dryness under a reduced pressure. AMP, pyridine-water (10 : 1, by volume) and DCC were measured into the reaction flask and a glass bead was added. The whole was vigorously shaken on a mechanical shaker at room temperature. At the times indicated a portion of the reaction mixture was removed and chromatographed on paper strips and radioautograms were prepared. Although a spot density on radioautogram is not necessarily proportional to the intensity of radioactivity of the spot area, the percentage of each spot can be roughly estimated by this method. Percentage of iP^{32} converted to ADP^{32} was calculated by measuring the density of each spot on the radioautograms with a densitometer (Toyorika Instrument Inc.). Density of the spot of ADP^{32} on one radioautogram prepared from a paper chromatogram had the same percentage as that of the spot density of ADP^{32} on another radioautogram from a chromatogram by a different solvent system. Accordingly, the spot having the R_f of ADP on chromatogram may be considered to contain ADP alone. Therefore, best conditions for obtaining a high yield of P^{32} were examined using the percentage of the spot density of ADP^{32} on radioautogram as the method of determination.

The reaction systems contained the following constituents: H_3PO_4, 0 (expt. I), 0.2 (expt. II), 2.0 (expt. III) and 10.0 μmoles (expt. IV); iP^{32}, about 20 μc.; AMP, 20.0 μmoles; pyridine-water (10 : 1, by volume), 0.5 ml.; and DCC, 500 μmoles. The reaction flasks were shaken at room temperature ($20 \pm 2°$). Portions of the reaction mixtures were removed at 0, 1, $2\,^1/_4$ and 5 hour intervals from the initiation of the shaking and chromatographed. As for DCC used in these experiments, no difference was observed between two preparations of DCC, one of which was kindly prepared by

Dr. K. Wakabayashi in this laboratory according to Lowenstein's method (*4*) and the other was a commercial product of the Daiichi Pure Chemicals Co., Ltd. The percentage of iP^{32} converted to ADP^{32} seemed to reach maximum in about 2 hours under the conditions of experiment I, as shown in Table III.

TABLE III

Relationship between the Reaction Time and the Distribution of P^{32} in the Products Formed during the Synthesis of ADP^{32} (Measured by Each Spot Density on the Radioautograms)

The reaction systems constained the following constituents: H_3PO_4, 0 (expt. I), 0.2 (expt. II), 2.0 (expt. III), and 10.0 μmoles (expt. IV); iP^{32}, about 20 μc.; AMP, 20.0 μmoles; pyridine-water (10 : 1, by volume), 0.5 ml.; and DCC, 500 μmoles. The reaction flasks were shaken at room temperature (20±2°). Portions of the reaction mixtures were removed at 0, 1, 2 1/4 and 5 hour intervals from the onset of the shaking and chromatographed with the solvent system of butyric acid-ammonia-EDTA. Radioautograms were prepared from the chromatograms. The values given are the percentage of each spot density to the total density of each run on the radioautograms.

Experiment number	Reaction period (*hour*)	R_f		
		0.54	0.60	0.74
		Substance corresponding to each R_f		
		ATP, iP	ADP	—
I	0	100		
	1	23	66	11
	2 1/4	19	68	13
	5	21	65	14
II	0	100		
	1	27	65	8
	2 1/4	32	60	8
	5	28	58	14
III	0	100		
	1	46	39	15
	2 1/4	45	43	12
	5	50	41	9
IV	0	100		
	1	58	34	8
	2 1/4	48	46	6
	5	51	43	6

In order to ascertain this phenomenon, an experiment was carried out under the same conditions as those of experiment I, except the reaction period. Following separation of the reaction products by paper chromatography, the radioactive substances in each spot were eluted with 1 *N* HCl from the filter paper and counted. The percentage of radioactivity of the substances in each spot to the total activity was calculated (see Table IV).

In this case it was revealed that the optimum length of time for the reaction was also 2 hours. Thus it was decided to carry out the synthesis for 2 hours without the carrier, H_3PO_4.

TABLE IV

Relationship between the Reaction Time and the Distribution of P^{32} in the Products Formed during Synthesis of ADP^{32} (Measured by Radioactivity of Each Spot on the Paper Chromatograms)

The reaction mixture consisted of about 20 μc. (carrier free) of iP^{32}, 20 μmoles of AMP, 0.5 ml. of pyridine-water (10 : 1, by volume), and 500 μmoles of DCC. The reaction flask was shaken at room temperature ($20\pm2^\circ$). Portions of the reaction mixture were removed at 0, 2/3, 2 and 9 hour intervals from the onset of the shaking and chromatographed with the solvent system of butyric acid-ammonia-EDTA. The values given are the percentage of radioactivity of each spot to the total activity of each run on the paper chromatogram.

Reaction period (*hour*)	R_f				
	0.48	0.54	0.60	0.74	0.81
	Substance corresponding to each R_f				
	PP	ATP, iP	ADP	—	—
0		100			
2/3		52	41	7	
2		29	58	13	
9	1	28	46	15	10

(*ii*) *Procedures for the Synthesis*—A similar reaction mixture as mentioned above (experiment I) was shaken at room temperature for 2 hours, except that 1 mc. of iP^{32} was used. After addition of 1.0 ml. of water, the mixture was shaken rapidly for 1 minute. The flask was centrifuged to separate the precipitate, which was washed three times with water. The supernatant and washings were combined and pyridine remaining in the solution was extracted three times with 4.0 ml. of ether. After addition of 30 μmoles of ADP as a carrier to the solution, it was diluted with about 100 ml. of water, adjusted to pH 8.0 with NaOH, and drawn through a 5.0×0.6 cm. column of Dowex-1 in the Cl^- form to adsorb the reaction products.

The results of separation of products by the column chromatography are shown in Fig. 2. There appeared five peaks (I-V) with absorption at 260 mμ. The first peak (I) was not identified and no attempt was made to clarify its nature. The second (II) seemed to correspond to a mixture of residual AMP and that which contaminated the carrier ADP preparation, as the peak appeared as the same position as that of AMP and had no radioactivity. The third (III) seemed to represent a symmetric nucleotide of the formula $(AMP)_2$, because it had absorption at 260 mμ without radioactivity. The fourth (IV) was a peak of ADP. The fifth (V) was considered to be composed of ATP and other unknown substances. Three peaks (A-C) with radioactivity appeared in the chromatogram. The first peak (A) was accounted for as the remaining iP^{32}. The second (B) with radioactivity coincided entirely with the peak of optical density corresponding to ADP. When a portion of the fraction was chromatographed on two paper strips by two different solvent systems, one of which was butyric acid-ammonid-EDTA and the other was isopropanol-ammonia-water (7:1:2, by volume) of Markham and Smith (*16*), and the radioautograms were prepared, the spot on each paper chromatogram agreed fully with that on the corresponding radioautogram and had the same R_f as that of ADP. The third (C) was considered to contain ATP^{32} and other unknown substances with radioactivity by the position of elution and the results of paper chromatography.

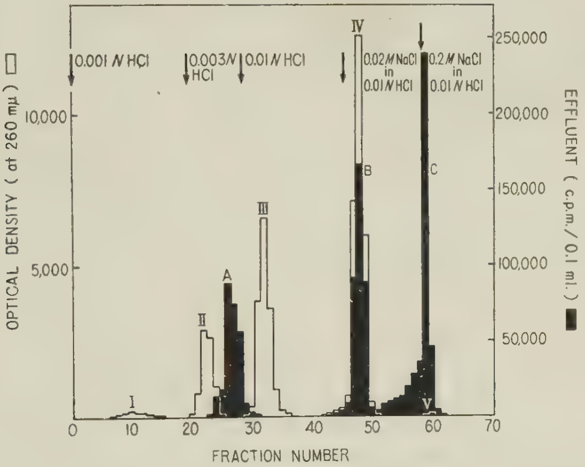


FIG. 2. Separation of the products formed during the synthesis of ADP^{32}. Exchanger: Dowex-1 ($\times$ 4 per cent)-Cl$^-$, 200–400 mesh, 5.0$\times$0.6 cm. Eluting solution: HCl, NaCl as shown. Fraction volume: 10 ml. Flow-rate: 2 ml./minute.

The fraction of ADP^{32} was collected and concentrated followed by the precipitation of Ba salt of ADP^{32} as described in the preparation of ATP-γ-P^{32}.

(*iii*) *Results and Discussion*—Ba-ADP^{32} obtained was 22 μmoles. The precentage of radioactivity of ADP^{32} to total activity of the effluent from the column was 35 per cent. Although there remained a small amount of substances with radioactivity on the column after elution with 0.2 M NaCl in 0.01 N HCl and a part of ADP^{32} was lost during the process of collection of the ADP as a Ba salt, the yield of ADP^{32} was regarded to be at least above 25 per cent in terms of P^{32}.

It has been reported that mitochondria converted 90 per cent of iP^{32} to ATP^{32} by using the oxidative phosphorylation system (*17*). Even if the yield of ADP^{32} which is obtained by splitting iP^{32} from the ATP^{32} is only 65 per cent based on P^{32}, this method of preparing ADP^{32} with mitochondria is better than that with DCC with regard to recovery of P^{32}. Nevertheless, the method of using DCC has the advantage of enabling one to obtain ADP^{32} which has a higher specific radioativity than that of any ADP^{32} prepared by other procedures known up to the present, so long as the amount of ADP added as a carrier is decreased.

(2) *Enzymic Step*

(*i*) *Procedures for the Synthesis*—Ba-ADP^{32} prepared by the above method was dissolved in cold 0.1 *N* HCl. After removing Ba^{++} by addition of a calculated amount of H_2SO_4 and centrifugation, pH of the solution was adjusted to 7.5 with KOH. The reaction mixture for the synthesis of ATP^{32} consisted of: 6 μmoles of ADP^{32}, 12 μmoles of PEP, 15 μmoles of $MgSO_4$, 150 μmoles of KCl, 30 μmoles of KF, 15 μmoles of EDTA, 120 μmoles of Tris buffer, pH 7.5, and pyruvate kinase in final volume of 3.0 ml. The mixture was incubated at 37° for 30 minutes. Pyruvate kinase used in this step was prepared from rabbit muscle as a „crystalline enzyme" by the method of Bücher and Pfleiderer (*18*). According to the procedures of Baer (*19*), PEP was prepared as a Ag-Ba double salt. Immediately prior to use, Ag and Ba were precipitated with HCl and H_2SO_4, followed by neutralization of the solution with KOH. KF and EDTA were added to prevent the randomization of the labeling pattern due to the actions of ATPase and adenylate kinase which might contaminate the enzyme preparation. ATP^{32} produced was isolated as a Na salt by the procedures similar to that mentioned in the enzymic step of the preparation of ATP-P^{32}.

(*ii*) *Results and Discussion*—Na salt of ATP-β-P^{32} obtained was about 4.3 μmoles. Calculated from the values of optical density in the column chromatogram for the separation of the ATP^{32}, it was ascertained that about 94 per cent of substrate ADP^{32} was utilized for synthesis of ATP^{32}. Since the yield of ATP-β-P^{32} actually obtained was about 70 per cent in terms of ADP^{32}, the decrease by about 30 per cent was ascribed to loss at the stage of isolation of the ATP^{32}. The yield was *ca.* 18 per cent, based on P^{32}. The specific activity was 157, 000 c.p.m. per μmoles. The enzymic hydrolyzation analysis described below showed that the ratio of specific activities of α-P, β-P and γ-P was 2:70:1. The values given by the method for analysis were accurate to *ca.* 2 per cent; the deviation accounted for the slight randomization of labeling pattern of ATP^{32}.

IV. Analytical Methods

To determine the labeling pattern of ATP^{32}, two analytical methods were devised. In one method the specific activity of each phosphorus atom in ATP^{32} was calculated from the extinctions and the radioactivities of the partially hydrolyzed products of the ATP^{32}. In the other, γ-P or β-P and γ-P of ATP^{32} were liberated separately by enzymic means and then each specific activity was determined by the quantities of iP liberated and their radioactivities.

(*1*) *Partial Hydrolyzation Method by Heating*

(*i*) *Procedures*

a. Specific Activity of ATP^{32}—The specific activity was computed from optical density and radioactivity of the solution of ATP^{32} in 0.01 *N* HCl.

b. Specific Activity of Each Phosphorus Atom—After addition of a small amount of authentic ATP of the ATP^{32} solution used above, the fraction of ATP was obtained by column chromatography as mentioned in the section on preparation. ATP^{32} in the fraction was adsorbed on another small column (3.0×0.6 cm.) and eluted with *N* HCl. The test tube containing the concentrated ATP^{32} solution was immersed in a water bath at 100° for 2 minutes. The products of the hydrolysis were separated by the use of a Dowex-1 column to determine the optical density and radioactivity. The specific activity of α-P was calculated from the values of AMP fraction. By a similar calculation the specific activity of α- and β-P, and that of α-, β- and γ-P were obtained from the values of ADP and ATP fraction, respectively. The ratio of specific activities of three phosphorus atoms was calculated from these values.

(*ii*) *Discussion*—This analytical method requires a relatively long time, and it is difficult to measure many samples at the same time unless a number of instruments are available. Notwithstanding, these inconveniences are outweighed by the advantage that the values obtained by this method have very small errors.

(*2*) *Enzymic Hydrolyzation Method*

There is usually a large probability that the sample of ATP^{32} contains iP and ADP. In such a case accurate values can not be expected by the method mentioned above, if the sample is not purified prior to the analysis. In order to avoid such an inconvenience, the amount and the redioactivity of each P were evaluated, after iP at each position had been individually split from the sample of ATP^{32} and separated from other parts. The method of Bowen and Kerwin, (*20*) for determination of ADP and ATP with myosin was utilized to split γ-P alone from ATP^{32} on the one hand and both β- and γ-P on the other. When γ-P alone was liberated from ATP, EDTA was added to inhibit the adenylate kinase activity and accelerate the ATP-ase activity of myosin, whereas in the case of liberating β-and γ-P together $CaCl_2$ was used to accelerate the activity of adenylate kinase in the enzyme preparation. To seperate iP from organic phosphate and evaluate iP Takahashi's modification (*21*) of the method of Beremblum and Chain (*22*) was employed, using isobutanol to extract iP and ascorbic acid as the reducing reagent.

(*i*) *Reaction of Splitting iP from ATP*—The following reaction conditions were in accordance with those of Bowen and Kerwin. (*20*). Myosin was prepared from rat muscle by the modification of Bowen and Kerwin (*20*) of Szent-Györgyi's method.

a) Cleavage of γ-P

Reaction mixture	(Final conc.)
Sample ATP^{32}	
KCl	0.7 *M*
EDTA	0.004 *M*
Myosin	
Tris buffer, pH 8.0	0.04 *M*
Total volume	1.25 ml.
Incubation temperature	27°
Incubation period	30 minutes

b. Cleavage of β- and γ-P

Reaction mixture	(Final conc.)
Sample ATP^{32}	
KCl	0.1 *M*
$CaCl_2$	0.005 *M*
Myosin	
Tris buffer, pH 8.0	0.04 *M*
Total volume	1.25 ml.
Incubation temperature	27°
Incubation period	30 minutes

The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 8 per cent.

(*ii*) *Extraction, Evaluation and Counting of iP*^{32}—From the deproteinized solution iP was extracted with isobutanol and determined by Takahashi's modification (*21*) of the method of Beremblum and Chain. (*22*). Isobutanol was previously saturated with water to prevent the change of volume of both isobutanol and water layers, when isobutanol was shaken with the sample solution. Radioactivities of both isobutanol and water layers were separately counted, after evaporation of aliquots to dryness on counting discs. Radioactivities per μatom of β-and γ-P, γ-P and α-P were computed from these values obtained by the above method. Using the radioactivities per μatom here obtained, the specific activity of each phosphorus atom could be easily calculated.

(*iii*) *Discussion*—About 2 per cent iP remains unextracted after extraction with isobutanol under the conditions described in section (*ii*) as reported by Yoshizawa (*23*), but this procedure needs no special instruments and several samples can be evaluated simultaneously in a short period of time. In spite of the error, this method is considered to be satisfactory for practical use, depending on the purpose of evaluation.

SUMMARY

1. A method was described for the preparation of ATP-γ-P^{32} and ATP-β-P^{32} with high specific activities and marked differences in radioactivity between each phosphorus atom sufficient for practical use. The convenient methods for analysis of ATP^{32} regarding the labeling pattern were also devised.

2. The method for preparation of ATP-γ-P^{32} consisted of chemical and enzymic steps. The ATP^{32} was synthesized by transferring iP^{32} to ADP from carbamyl phosphate-P^{32}, which was prepared with orthophosphate-P^{32} and KCNO, using carbamyl phosphate kinase. Since the yield in terms of P^{32} depends on the yield of carbamyl phosphate in the chemical step, the reaction conditions of this step were studied extensively.

3. The method for preparation of ATP-β-P^{32} was also composed of a chemical step, in which ADP^{32} was synthesized from orthophosphate-P^{32} and AMP with dicyclohexylcarbodiimide, and a enzymic step, in which ATP^{32} was prepared by the transphosphorylation from phosphoenolpyruvate to ADP^{32} catalyzed by pyruvate kinase. In order to increase the overall yield, the reaction conditions in the chemical step were investigated.

4. Concerning the analysis of the labeling pattern of ATP^{32}, two methods were described. In one method the specific activity of each phosphorus atom in ATP^{32} was computed from the extinctions and the radioactivities of the products partially hydrolyzed by acid treatment. In the other, the amount and the radioactivity of each phosphate in ATP^{32} were determined, after liberation individually by means of ATPase and adenylate kinase in the myosin preparation and extraction separately with isobutanol. The specific activity of each phosphorus atom was calculated from the values thus obtained.

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A BRANCHED SATURATED C_{15} ACID (SARCINIC ACID) FROM SARCINA PHOSPHOLIPIDS AND A SIMILAR ACID FROM SEVERAL MICROBIAL LIPIDS

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As a result of our studies on microbial lipids a special type of fatty acid referred to as "Sarcinic acid" for convenience' sake was found to occur in the phospholipid fraction of lipids from a certain strain of *Sarcina*. The phospholipid has proved to be a mere phosphatidic acid which contains this acid as a sole fatty acid component. Sarcinic acid was identified as a saturated liquid acid possessing C_{15} carbon chain and characterized by its optical activity.

Further investigations made in this line have provided evidence that an acid similar to or identical with sarcinic acid should exist in the lipids of other microorganisms such as *B. subtilis*, and *B. natto*.

In the present paper are reported the isolation and characterization of this fatty acid, the detailed description of which will be presented below.

EXPERIMENTAL AND RESULTS

Bacterial Material—The organism was grown on a plain bouillon agar plate at 37° for 5 days. At the end of the term the culture was washed out from the plate with water which had been made acidic with HCl to pH 4.0. The aqueous bacterial suspension was centrifuged, the bacterial cell material thus obtained in a paste form, after four times washing with water, was desiccated *in vacuo* and the dried matter was powdered employing a stainless steel ball mill.

Extraction of Phospholipids—430 g. of this bacterial powder were extracted with five times its weight of an ethanol-ether mixture (1 : 1) at room temperature with occasional shaking for five days. This extraction procedure was repeated two more times. The combined extract was evaporated to a small volume to remove most of the organic solvents and the residue was taken up in ether. The ether solution, after washing with water, was concentrated to an appropriate volume, mixed with four times its volume of cold acetone, when crude phospholipids precipitated in an amount of 4.38 g., the yield corresponding to 1 per cent on a dry weight basis of bacteria. The crude phospholipids gave a yellow brown soft solid, melting at 189–190° and having 4.16 per cent P and 1.71 per cent N. The supernatant solutions separated by centrifugation from the phospholipids were combined, evaporated to dryness to obtain a crude acetone soluble fraction as a dark brown liquid of 4.02 g., corresponding to 0.9 per cent of dried bacteria.

Subfractionation of Crude Phospholipids into P-I, P-II, and P-III Fractions—4.30 g. of crude phospholipids were boiled with 30 ml. ethanol and then cooled at 0°. The mixture was separated into a cold ethanol insoluble fraction P-I and a soluble fraction. The latter, upon distilling off the ethanol, was dissolved in 5 ml. ether and poured into 50 ml. ice cold acetone to precipitate an acetone insoluble fraction P-II. The filtrate from P-II gave an acetone soluble fraction P-III. Yield and analytical data are as follows:

P-I (3.5 g., P 4.58 per cent, N 1.71 per cent);
P-II (0.38 g., P 3.67 per cent, N 0.72 per cent);
P-III (0.40 g., P 4.02 per cent, N 0.46 per cent);

Crude Phosphatidic Acid from P-I Phospholipids—3.1 g. of P-I fraction were dissolved in 40 ml. of ether, shaken with an equal volume of 0.5 *N* HCl and the ethereal layer was washed several times with water, until the separation of ethereal and aqueous layers became difficult due to gel formation. The ethereal solution was evaporated to form a water insoluble residue which was then taken up in ether and from the ethereal solution, after drying with Na_2SO_4, the ether was distilled off, and a crude phosphatidic acid obtained in an amount of 2.4 g. with 0.10 per cent N and 4.7 per cent P. The 0.5 *N* HCl used for washing the ethereal solution of P-I was evaporated to dryness *in vacuo.* Thereby was obtained a slight amount of residue which proved to be almost entirely an inorganic mixture consisting of NH_4Cl and $MgCl_2$ and to be free of organic bases such as choline and ethanolamine. This indicated that the nitrogen found in the original phospholipids might have originated from ammonia existed in the lipids, in other words, the lipids being studied deal with the ammonium as well as the magnesium salt of phosphatidic acids and not with the ordinary phospholipids which contain organic bases as constituents.

Sarcinic Acid from Phosphatidic Acids—2.1 g. of the crude phosphatidic acids were hydrolyzed by boiling with 5 per cent H_2SO_4 for 8 hours. The hydrolysate was extracted with ether, the ethereal solution was evaporated to dryness to obtain fatty acids in amounts of 1.4 g. the yield corresponding to 66 per cent of the lipid material used. The free acids were converted into methyl ester by boiling with methanol containing HCl and the ester was subjected to fractional distillation in a high vacuum, whereby almost all of the ester distilled at 85–90°/1×10^{-4} mm. Hg. The ester was saponified to yield 1.30 g. of sarcinic acid.

Properties of Sarcinic Acid—This acid formed a colorless liquid at ordinary temperature. A crystallized sample melted at 24°. Iodine number was found zero. Specific rotation of methyl ester gave $[\alpha]_D^{27} = +5.26°$. Molecular weight from neutralization number was estimated at 241, the theoretical value for $C_{15}H_{30}O_2$ being 242. Elemental analysis gave C 74.35 (74.32) per cent, H 12.37 (12.48) per cent, wherein the figures in parenthesis being the theoretical values for the above formula.

Isolation of α-Glycerophosphate as Barium Salt from the Hydrolysate of P-I—The aqueous solution obtained by ether extraction of the H_2SO_4 hydrolysate of P-1 was neutralized with $Ba(OH_2)$ and $BaSO_4$ was filtered off. The solution was concentrated and ethanol was added to 80 per cent when a precipitate of barium salt occurred. Chromatographic analysis showed that the salt consisted mostly of α-glycerophosphate.

Sarcinic Acid from the Acetone Soluble Lipid Fraction (*Sarcina Fats*)—3.8 g. of acetone soluble lipids were saponified with alcoholic potash and separated according to the ordinary procedure into 52.9 per cent mixed fatty acids and 40.0 per cent crude glycerol. The mixed fatty acid fraction was separated by the use of the lead soap ether-ethanol technique into an insoluble and a soluble lead soap fraction. After removal of the lead metal the both fractions were converted into methyl ester respectively, leading to two preparations A and B. A represented the methyl ester from the insoluble lead soap fraction, yield 0.613 g. (28.2 per cent of the original fat used), and B represented the methyl ester from the soluble lead soap fraction, yield 0.525 g. (23.7 per cent). The total amounts of both A and B were subjected to fractional distillation in a high vacuum respectively, the data of which are given below.

Preparation A:

Fraction I	(90–110°/1×10^{-4} mm.)	0.561 g.
,, II	(110–130°/1×10^{-4} mm.)	0.025 g.
,, III	residue	a trace

Fraction I, upon saponification, liberated an acid identical with sarcinic acid, showing such characteristics: I No. 0, molecular weight 240, and m.p. 24°.

Preparation B:

Fraction I	(115–130°/6×10^{-4} mm.)	0.427 g.
,, II	(130–160°/6×10^{-4} mm.)	0.025 g.
,, III	residue	a trace

Upon saponification, Fraction I gave an acid characterized by those properties, which was quite close to sarcinic acid, *i.e.*, I No. 3.2, molecular weight 239, and m.p. 21.5°.

Sarcinic Acid-like Substance from the Acetone Soluble Lipids of B. subtilis and B. natto—B. subtilis: Ethanol-ether extract of 250 g. of dried, ground bacterial cells gave a lipid material which was then separated into an acetone soluble (2.0 per cent) and an acetone insoluble fraction (0.8 per cent), the figures denoting the percentage of yields based on the dried bacillus material. The acetone soluble lipids were saponified to obtain free fatty acids. The latter compounds were converted into lead soaps, separated into ether soluble and insoluble fractions and each fraction was converted into methyl ester, giving two preparations A and B. A represented 1.510 g. of methyl ester (28.3 per cent of the original fat used) from the insoluble lead soap fraction and B represented 2.972 g. of methyl ester (32.5 per cent) from the soluble lead soap fraction. The fractional vacuum distillation of A and B gave the following results.

Preparation A:

Fraction I	(95–110°/1×10^{-3} mm.)	1.05 g.
,, II	(110–120°/1×10^{-3} mm.)	0.36 g.
,, III	residue	a trace

Upon saponification, Fraction I separated a substance which gave I No. 0.0, molecular weight 246, and m.p. 39°, pointing to the presence of a mixture of a C_{15} acid possibly sarcinic acid and a C_{16} acid.

Preparation B:

Fraction I	(100–110°/1×10^{-3} mm.)	2.360 g.
,, II	(120–140°/2×10^{-3} mm.)	0.156 g.
,, III	(150–170°/4×10^{-3} mm.)	0.217 g.
,, IV	residue	a trace

Fraction I was saponified to yield a substance which gave I No. 32.5, molecular weight 247, and m.p. 29°, indicating the presence of a probable mixture of C_{15} and C_{16} acids.

B. natto: Ethanol-ether extract of 250 g. of dried, ground bacterial cells yielded a lipid material which was separated into an acetone soluble (2.1 per cent) and an acetone insoluble fraction (1.1 per cent). The acetone soluble lipids were saponified to yield free fatty acids. From the fatty acids two preparations A and B were obtained as described above. A represented 0.425 g. of methyl ester (16.0 per cent) from the ether insoluble lead soap fraction and B represented 1.628 g. of methyl ester (52.7 per cent) from the ether soluble lead soap fraction. Each fraction was fractionated by vacuum distillation giving the results as follows.

Preparation A:

Fraction I	(100–120°/6×10^{-4} mm.)	0.400 g.
,, II	residue	a trace

Free acids from Fraction I gave I No. 0.0, moleculer weight 247, and m.p. 32°, indicating the presence of a probable mixture of C_{15} and C_{16} fatty acids including sarcinic acid.

Preparation B:

Fraction I	(85–100°/1×10^{-3} mm.)	0.320 g.
,, II	(100–120°/1×10^{-3} mm.)	1.016 g.
,, III	(120–145°/1×10^{-3} mm.)	0.014 g.
,, IV	(145–175°/1×10^{-3} mm.)	0.085 g.
,, V	residue	a trace

Free fatty acids from the main Fraction II gave I No. 11.52, moleculer weight 248 and m.p. 26°, indicating the presence of a probable mixture of C_{15} and C_{16} fatty acids with some unsaturated bond.

Analysis of Sarcinic Acid and its Allied Compounds by Reversed-Phase Partition Chromatography—The technique made available by Howard and Martin (*1*) and elaborated by Silk and Hahn (*2*) was employed for differentiating sarcinic acid from other closely related fatty acids. A column (1×100 cm.)

was loaded with a suspension of about 28 g. of paraffine coated siliconized Hyflo-Super-Cel (Wako) in a suitable amount of 55 per cent aqueous acetone saturated with liquid paraffine. Elution of samples was conducted with a series of aqueous acetone in an increasing order of concentration such as 60, 65, and 70 per cent and at a flow rate of about 40–50 ml. per hour. Each 2

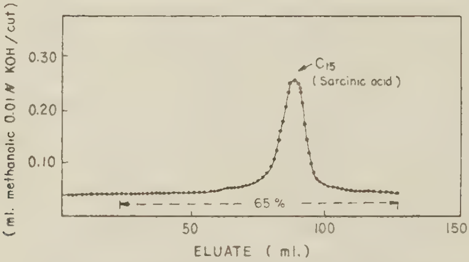

FIG. 1. Chromatogram of sarcinic acid. Sample applied, 6 mg. in 55 per cent aqueous acetone. Recovery, 90 per cent. This diagram shows apparently a sharp single peak accounting for the presence of a unique compound.

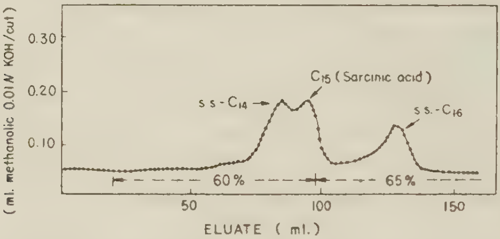

FIG. 2. Chromatogram of a synthetic mixture consisting of 10 μM myristic (s.s.-C_{14}), 3 mg. sarcinic (C_{15}), and 10 μM palmitic acid (s.s.-C_{16}). This figure indicates a possible resolution of the mixture consisting of nearly equimolecules of these three fatty acids.

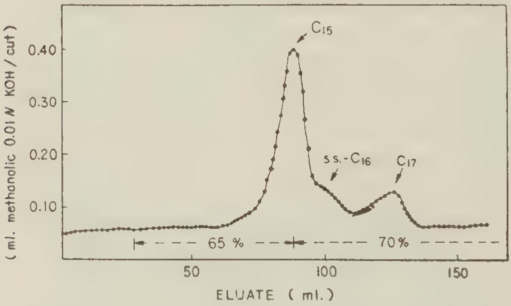

FIG. 3. Chromatogram of fatty acids from *B. natto*. Sample applied, 13 mg. in 55 per cent aqueous acetone. Recovery, 96 per cent. This chromatogram shows the presence of two small peaks possibly of palmitic (s.s.-C_{16}) and an unknown C_{17} acid in additon to a C_{15} acid.

ml. portion of eluates was collected and titrated with 0.02 N KOH in methanol using bromothymolblue as indicator. A variety of samples were examined: 1) sarcinic acid from sarcina; 2) a synthetic mixture of sarcinic acid (C_{15}) and known fatty acids such as myristic (s.s.-C_{14}) and palmitic (s.s.-

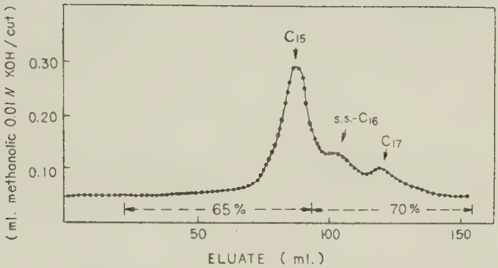

FIG. 4. Chromatogram of fatty acids from *B. natto* pretreated with $KMnO_4$, according to the Berthram oxidation procedure. No marked change in the shape of chromatogram is observable on comparison with Fig. 3, indicating the absence of unsaturated fatty acids in the sample being studied.

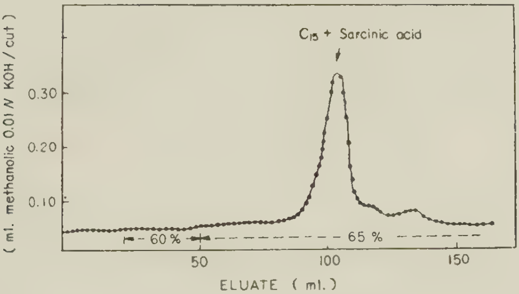

FIG. 5. Chromatogram of fatty acids from *B. natto* with added sarcinic acid. Sample applied, 6 mg. of fatty acids plus 4 μM sarcinic acid. C_{15} peak coincides well with that of sarcinic acid, suggesting the identity of the two fatty acids.

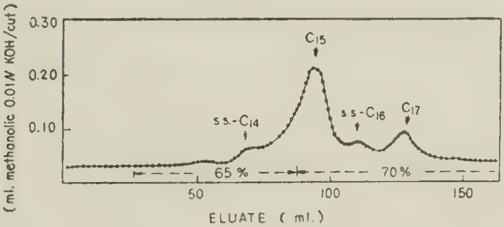

FIG. 6. Chromatogram of fatty acids from *B. subtilis.* Sample applied, 15 mg. Recovery, 96 per cent. Four peaks are revealed suggesting the presence of myristic, palmitic, C_{15} and C_{17} acids.

C_{16}) acid: 3) fatty acids from *B. natto* (Fraction II of preparation B); 4) the same sample as 3) pretreated with $KMnO_4$; 5) the same sample as 3) with added sarcinic acid; 6) fatty acids from *B. subtilis* (Fraction I of preparation A).

The chromatograms obtained are illustrated in the above.

DISCUSSION

From the analytical figures presented in this paper involving the iodine number, molecular weight, melting point and optical activity, the sarcinic acid is to be considered a kind of branched pentadecanoic acid. In support of this view is its chromatographic behavior, according to which sarcinic acid emerged in the chromatogram between the two peaks of myristic and palmitic acids, especially very close to the former. It is noticeable to find that this compound is a principal fatty acid isolated from both the acetone soluble and the insoluble lipid fraction and also that the main phospholipid, namely, Fraction P-I from sarcina lipids is the magnesium as well as the ammonium salt of phosphatidic acid, whose fatty acids consisting almost entirely of sarcinic acid. The ordinary method of separating liquid and solid high molecular fatty acids due to solubility difference of the lead soap in ether or alcohol has proved inadequate for the effective preparation of sarcinic acid, since this unique liquid saturated fatty acid was obtained from both the soluble and the insoluble lead soap fraction.

The possible occurrence of sarcinic acid or a like compound in *B. natto* and *B. subtilis* has been evidenced by the studies on their acetone soluble lipids. In either case the principal fatty acid obtained by fractional distillation of the methyl esters from soluble as well as insoluble lead soaps was found to be more or less similar in their analytical figures. They gave relatively low melting points within range between 21.5° and 39° (24°), mean molecular weights ranging from 239 to 247 (240) and nil or very small number of I No., the values required for sarcinic acid. Experiments carried out by chromatography on the samples with or without added sarcinic acid as a check demonstrated that all the samples examined were mixtures consisting either of C_{14}, C_{15}, C_{16}, and C_{17} acids or C_{15}, C_{16}, and C_{17} acids and in each case C_{15} acid was the dominating one.

Recent investigation made by Hansen *et al.* have shown that two isomeric C_{15} branched chain acids, namely, 13-methyltetradecanoic acid or the iso acid having the formula $CH_3(CH_3)(CH_2)_{11}COOH$ and 12-methyltetradecanoic acid or *anteiso* acid having the formula $CH_3CH_2CH(CH_3)(CH_2)_{10}COOH$ are occurring, though in trace amounts, in mutton fat (*3*) and butter fat (*4*). The anteiso acid showed m.p. 23.7°, sap. equiv. 242.2, iodine No. 0.0 (Wijs) and $[\alpha]_D^{18} = +4.8°$. These analytical data, which are in fair agreement with those found for sarcinic acid, seem to indicate that the two compounds would be identical, although the identity has not yet been confirmed by the mixed melting point procedure. Anyway, sarcinic acid or a like C_{15} acid has been established to abound in common bacteria as those mentioned before.

Moreover, in this connection it will be of interest to state that *B. natto* is used in Japan as fermenting agent in the manufacture of a Japanese familiar food called "natto" from soy bean. From the physiological viewpoint some of the lipid material liberated from intestinal microbes ought to be absorbed along with other food lipids to enter the tissue lipids. Accordingly, in case unusual fatty acids are encountered in minute amounts in the body lipids from herbivorous animals, as indicated to be case with mutton, ox, etc. it is possible that they have orignated partly from lipids of microorganisms residing enormously in the host gut.

SUMMARY

1. A C_{15} liquid saturated fatty acid (sarcinic acid) was isolated from *Sarcina* phospholipids.

2. The principal phospholipid fraction has proved to consist of the magnesium and the ammonium salts of phosphatidic acid, the sole fatty acid component of which being sarcinic acid.

3. The identical or a similar C_{15} acid was found to reside in the lipids of other microorganisms such as *B. subtilis* and *B. natto*.

4. Suggestion was made of the identity of sarcinic acid with 12-methyltetradecanoic acid occurring in mutton fat and butter fat.

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STUDIES ON SULFATASES OF CHARONIA LAMPAS. I.

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Sulfatases of the liver of a marine gastropod, *Charonia lampas* (*Tritonalia sauliae*), have been investigated since 1931 by Soda, Egami and their coworkers (*1*). Arylsulfatase*, glycosulfatase*, chondrosulfatase and myrosulfaminase (*2*)* were found by them in the extract of the liver.

The present report deals with the localization and some properties of sulfatases in the liver of the animal. Histological study on the organ is also included.

MATERIALS AND METHODS

Preparation of Substrates—2-Hydroxy-5-nitrophenyl sulfate (nitrocatechol sulfate, NCS) was prepared according to the directions by Roy (*3*) and purified by the method of Dodgson and Spencer (*4*).

Potassium *p*-nitrophenyl sulfate (*p*-NPS) was prepared by the method of Harada *et al.* (*5*) with slight modifications: 15 ml. Chlorosulfonic acid was added slowly to 17 ml. dry pyridine in 42 ml. dry chloroform at 0° with vigorous stirring and with strict prevention of moisture. To the resultant mixture, solution of 10 g. *p*-nitrophenol in 24 ml. dry pyridine was added. After stirring for 2 hours and standing for 1 hour, *p*-nitrophenyl sulfate was extracted from the reaction mixture with cold water. Most part of *p*-nitrophenol remained in the residue. The extract neutralized with potassium hydroxide at 0°, was evaporated *in vacuo* until it became dry. *p*-NPS was extracted from the residue by hot ethanol. Then two recrystallizations from 90 per cent ethanol gave small white needles in 60 per cent yield. The compound was free from SO_4'' and *p*-nitrophenol.

Glucose-6-sulfate (GS) was prepared as usual by sulfation of glucose with chlorosulfonic acid (6) and the quantitative separation of glucose monosulfate was achieved by ion exchange chromatography as follows; The aqueous solution of crude product containing calcium glucose-6-sulfate was passed through a column of an anion exchanger Dowex-1 (OH type). Unchanged glucose was removed with water. Subsequent elution with 0.03 *N* sulfuric acid gave glucose monosulfuric acid in separate fraction. Molisch's color reaction was used to detect carbohydrates in effluents. The pooled GS fraction of the effluents was neutralized with barium carbonate. By concentrating the filtrate *in vacuo*, barium glucose monosulfate was obtained by addition of ethanol. The barium salt of GS was converted into potassium salt by adding the requisite amount of potassium sulfate to a concentrated aqueous solution of the barium salt. The precipitated barium sulfate was removed by centrifuging and the potassium glucose monosulfate in the supernatant was precipitated with absolute ethanol. The compound appeared to be homogeneous on paper chromatogram (n-butanol : ethanol : water : ammonium water = 40 : 12 : 20 : 1).

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* These enzymes were called formerly phenosulfatase, glucosulfatase and myrosulfatase respectively.

Sinigrin $CH_2CHCH_2NC(OSO_3K)SC_6H_{11}O_5 \cdot H_2O$ was given from Dr. Z. Nagashima, University of Shizuoka.

Preparation of Sulfatases—20 per cent (*w/v*) suspension of acetone dried liver in water was incubated for 24 hours at 30° with a few drops of chloroform and centrifuged. To the supernatant acriflavin (Trypaflavin) was added to a final concentration of 1 per cent (*w/v*) and centrifuged (*7*). To the supernatant chilled ethanol was added, giving a final ethanol concentration of 70 per cent (*v/v*). The precipitate obtained from 20 g. acetone powder was dissolved in 50 ml. water. The solution was dialyzed overnight against running water and insoluble material was removed by centrifuging. The supernatant contained arylsulfatase, glycosulfatase and myrosulfaminase.

Determination of Enzyme Activity—Enzyme activity towards *p*-NPS and NCS was measured by spectrophotometric estimation of the respective liberated phenols (*3*), (*8*). Enzyme activity towards GS and sinigrin was determined by estimating liberated sulfate by the method of Egami and Takahashi (*9*) or Dodgson and Spencer (*10*). Except where otherwise stated, the activity of arylsulfatase was measured in the presence of 0.02 *M* tris(hydroxymethyl)aminomethane-maleate (tris-maleate) buffer pH 6.0 or tris (hydroxymethyl) aminomethane (tris) buffer pH 7.0 (*11*), with 0.01 *M* NCS or *p*-NPS. Final volume of reaction mixture was 0.8 ml. containing 0.1 ml. enzyme solution. Incubation was for 1 hour at 37° ; The glycosulfatase, in tris-maleate buffer or acetate buffer pH 5.7 (*11*), with 0.02 *M* GS. Final volume of reaction mixture was 1 ml. containing 0.2 ml. enzyme solution, for 2 hours ; The myrosulfaminase, in the same buffer pH 5.7, with 0.01 *M* sinigrin. Final volume of reaction mixture was 1 ml. containing 0.2 ml. enzyme solution and a few drops of chloroform, for 16 hours.

RESULTS

Localization of the Three Enzymes in the Liver of Charonia lampas—Fresh liver of *Charonia lampas* was homogenized with sucrose solution and fractionated by centrifuging according to the method of Schneider and Hogeboom (*12*). Assay of the whole suspension and the separated supernatant and debris against four substrates under the respective conditions showed that the bulk of the original activities for NCS, *p*-NPS, GS and sinigrin was present in the 100,000 × *g*. supernatant (Table I). Almost same results were obtained in 0.677 *M* sucrose medium (isotonic with sea water) with those obtained in 0.25 *M* sucrose.

Properties of Enzymes—(*A*) *pH-Activity*—pH-activity curves for the three enzymes in the preparations were shown in Fig. 1.

(*B*) *Mutual Relation among Substrates*—(*i*) *NCS and p-NPS*: *p*-NPS inhibited the hydrolysis of NCS. The mode of inhibition by *p*-NPS was found to be competitive one as illustrated in Fig. 2. Moreover ratio of phenols liberated from NCS and *p*-NPS by arylsulfatase was about 100 : 44.4, in various buffer solutions (acetate, citrate (*11*) tris and tris-maleate buffer, PH 6.0 and 7.0.

(*ii*) *NCS and GS*: Enzyme activity towards 10^{-3}–10^{-2} *M* NCS was not inhibited by 10^{-3} *M* of GS but a definite inhibition (about 25 per cent) was observed in the presence of 2.5×10^{-2} *M* of GS (Fig. 3).

Table I

Localization of Arylsulfatase, Glycosulfatase and Myrosulfaminase

Fresh liver was homogenized in 0.25 *M* sucrose solution and fractionated by centrifuging.

	Suspension of whole fresh liver		600×*g*. precipitate		8500×*g* precipitate		100,000×*g* precipitate		100,000×*g* supernatant		Recovery sum of each fraction	
Activity (μ moles of phenol liberated/*hour*/*g*. wet wt. of liver)	NCS	*p*-NPS	NCS	*p*-NPS	NCS	*p*-NPS	NCS	*p*-NPS	NCS	*p*-NPS	NCS	*p*-NPS
	385	118	14.2	15.3	24.1	19.1	10.2	6.8	345	102	393.5	143.2
% of activity of whole suspension	100	100	3.7	12.9	6.3	15.1	3.1	5.8	89.8	86.5	106.8	120.3
Relative activity	1.0	1.0	0.17	0.59	0.46	1.15	0.37	0.70	1.19	1.14		
Activity (μ moles of SO_4'' liberated/*hour*/*g*. wet wt. of liver)	GS	sinigrin	GS	sinigrin	GS	sinigrin	GS	sinigrin	GS	sinigrin	GS	sinigrin
	17.5	1.32	0	0	0	0	0	0	19	1.7	19	1.7
% of activity of whole suspension	100	100	0	0	0	0	0	0	108	129	108	129

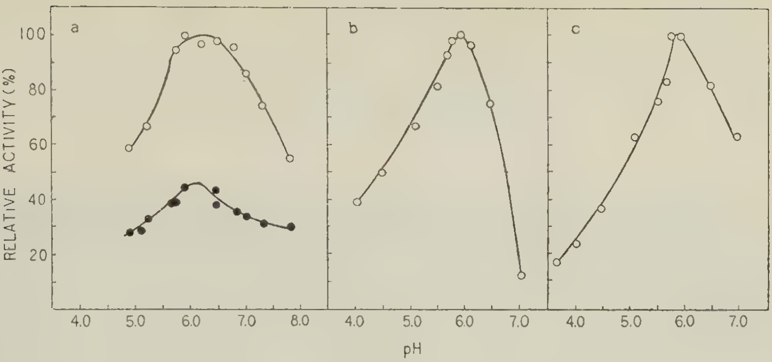


FIG. 1. pH-activity curves for arylsulfatase (a), glycosulfatase (b) and myrosulfaminase (c).

(a) —○— NCS (0.01 *M*); —●— *p*-NPS (0.01 *M*);
(b) —○— GS (0.02 *M*); (c) —○— sinigrin (0.01 *M*).

Enzyme activity was measured at 37° for 1 hour (a), 2 hours (b) and 16 hours (c) respectively, in the presence of 0.02 *M* acetate (pH 3.65–5.68) and tris-maleate (pH 5.08–7.8) buffers adjusted to the required pH. Maximum enzyme activity: (a) 18 μ moles nitrocatechol/ml. enzyme/hour, 7.3 μmoles *p*-nitrophenol/ml. enzyme/hour, (b) 3.7 μmoles SO_4''/ml. enzyme/hour, (c) 0.8 μmoles SO_4''/ml. enzyme/hour.

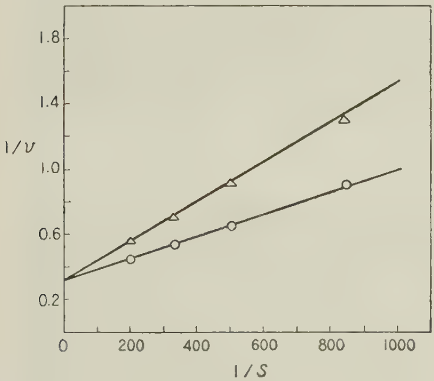


FIG. 2. Effect of substrate(NCS) concentration on activity and inhibition by *p*-NPS. Substrate concentration (S) expressed as 10^{-3} *M*. Reaction velocity (V) as 10^{-3} *M*/hour. —○— no inhibitor, —△— NCS+10^{-3} *M* *p*-NPS.

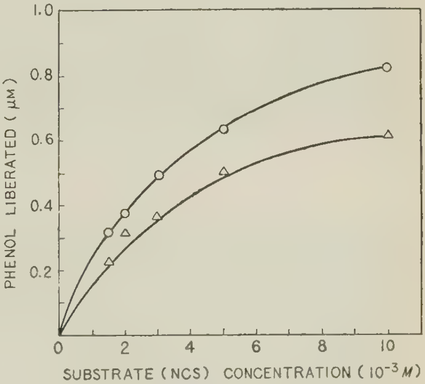


FIG. 3. Effect of GS on arylsulfatase. —○— no inhibitor, —△— NCS+2.5×10^{-2} *M* GS.

(*iii*) *NCS and Sinigrin*: Enzyme activity towards 0.01 *M* NCS was not inhibited by 0.1 *M* sinigrin.

(*iv*) *NCS and Inositol hexasulfate* (*13*): Enzyme activity towards 0.01 *M* NCS was not inhibitd by 0.1 *M* inositol hexasulfate.

(*v*) *GS and Inositol hexasulfate*: Enzyme activity towards 0.02 *M* GS was not inhibited by 0.2 *M* inositol hexasulfate.

(*C*) *Inhibitors*—Soda, Egami and their coworkers studied the effects of inhibitors on sulfatases, especially on glycosulfatase of the liver of *Charonia* (*14*). The behaviour of metal ions, negative ions, sugars and phenols towards glyco-

TABLE II

Effects of Inhibitors on the Activities of Arylsulfatase, Glycosulfatase and Myrosulfaminase

Activities were measured under the optimum condition for each enzyme as mentioned above.

Inhibitor		Percentage activity		
		Arylsulfatase	Glycosulfatase	Myrosulfaminase
No inhibitor		100	100	100
KH_2PO_4	10^{-3}	77.5	82.6	33.5
NaF	2×10^{-3}	93.5		0
	2×10^{-2}	57.0	71	0
Na_2SO_4	2×10^{-3}	92.5		
	2×10^{-2}	92.4		
KCN	2×10^{-3}	101	100	92
	2×10^{-2}	94	101	93
Ferricyanide	2×10^{-3}	58.7		
Maleic acid	2×10^{-3}	72	99.5	72.2
Iodine	10^{-1}	0	0	8.4
Iodoacetamide	2×10^{-3}	104	100	62
p-Chloromercuribenzoate	5×10^{-4}	115	106	66
	1.2×10^{-3}	106	110	
8-Hydroxyquinoline	5×10^{-3}	97	94	95
EDTA	10^{-2}	109	86	106

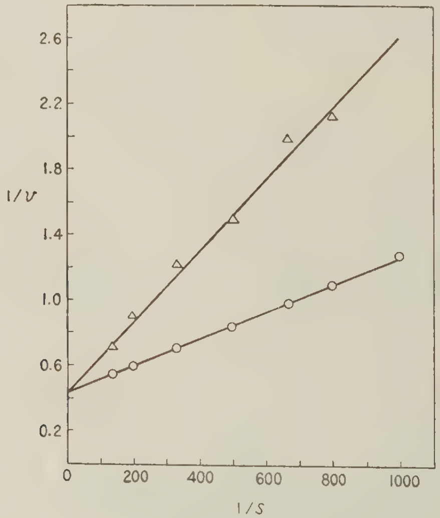
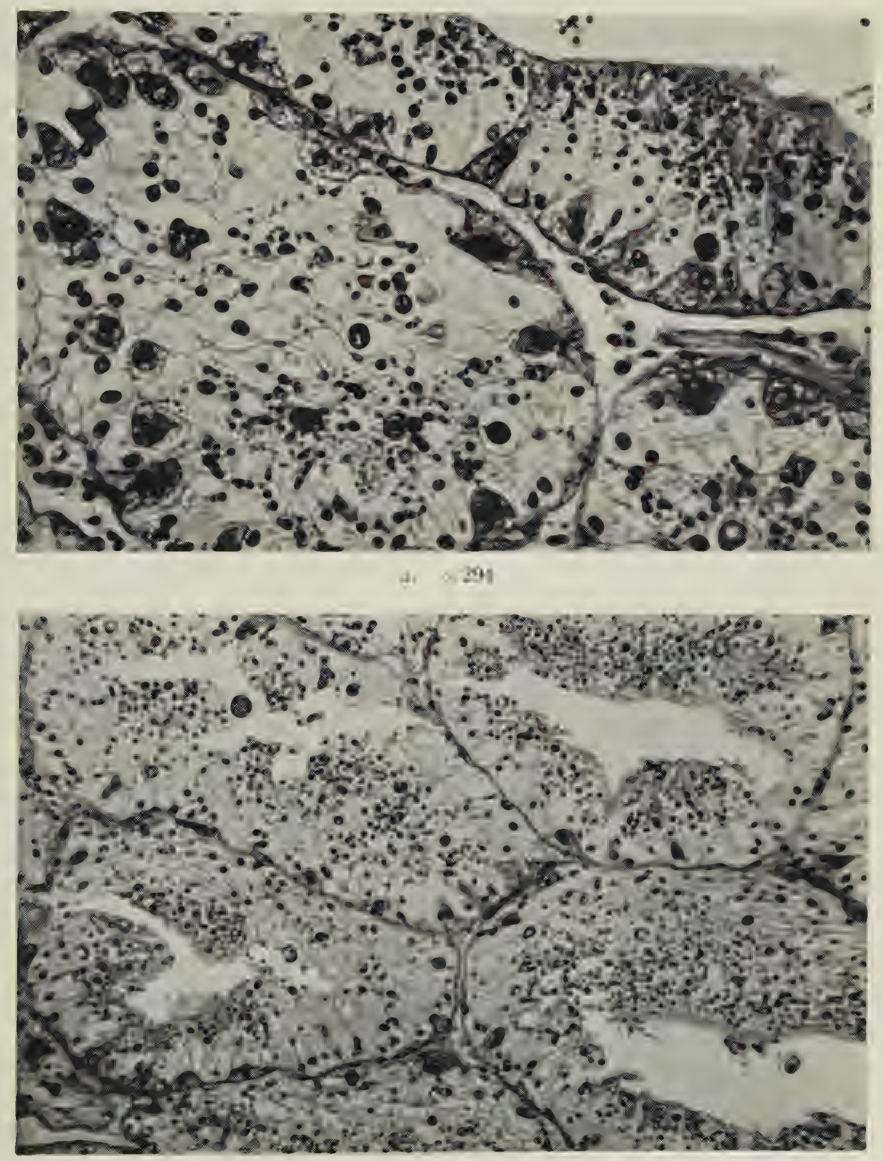


FIG. 4. Effect of substrate (NCS) concentration on activity and inhibition by phosphate. Substrate (NCS) concentration (S) expressed as 10^{-3} *M*. Reaction velocity (V) as 10^{-3} *M*/hour. —○— no inhibitor, —△— NCS+ 10^{-3} *M* phosphate.

sulfatase were described by them. Further studies on the effects of inhibitors on various sulfatase of *Charonia* were here again undertaken. These three enzymes were inhibited by phosphate and fluoride but were uninfluenced by cyanide, 8-hydroxyquinoline and ethylendiamine tetraacetic acid disodium (EDTA). (Table II.)



a. ×294

b. ×147

FIG. 5. Microphotograph of paraffin section of the liver of *Charonia lampas* stained with hematoxylin-eosin.

(*D*) *Competitive Inhibition by Phosphate*—The mode of inhibition of arylsulfatase by phosphate investigated according to Lineweaver-Burk's plot was found to be competitive as illustrated in Fig. 4. The hydrolysis of GS (0.002–0.04 M) and sinigrin (0.002–0.02 M) was also inhibited competitively by 0.001 M phosphate.

Histological Survey—Histologial survey of the liver of *Charonia* was carried out with various specimens. Fresh liver of *Charonia* was fixed by Bouin's fluid or absolute ethanol or Regaud's fluid. Paraffin sections were made successively and stained with hematoxylin-eosin, methyl green-pyronin, Lillie's reagent for polysaccharides and iron-hematoxylin for mitochondria respectively.

In the liver of *Charonia*, two types of liver cells were observed. The majority of liver cells, namely 40–50 high columnar cells with oval nuclei, formed glandular structure with surrounding blood capillary space. In the edge of these cells or lumina of these glandular structure, there were seen abundant secretory granules stained with Lillie's polysaccharide stain. On the contrary, mammalian bile pigment were negative to this staining method. Moreover, in these liver cells of *Charonia*, there were rarely seen mitochondria and RNA granules. This result was also different from the histological findings of mammalian (human being, rabbit or albino rat) liver cells, namely, in the cytoplasm of the latter, abundant mitochondria and RNA granules were contained.

Another type of liver cells of *Charonia* were also seen in the glandular structure described above. 3–5 cubic or pear shaped cells with large oval nuclei and abundant chromatin, were seen in the near basement membrane of the glandular structure. The minority of liver cells containing abundant fine mitochondria and RNA granules in their cytoplasm were similar to ordinary mammalian liver cells.

DISCUSSION

Investigation on the arylsulfatases of mammalian tissues have recently resulted in the recognition of two distinct types of arylsulfatases (*15*).

Type I: These enzymes have appreciable activity towards NPS but are much less active towards NCS. They are strongly inhibited by cyanide but are hardly affected by phosphate, sulfate and fluoride.

Type II: Enzymes of this type show high affinity and activity towards NCS but have a very low affinity and activity towards NPS. They are scarcely affected by cyanide but are strongly inhibited by phosphate, sulfate and fluoride.

On the contrary, arylsulfatase of the liver of *Charonia* have appreciable activity towards both NCS and *p*-NPS (100 : 44.4). Since NCS activity has been competitively inhibited by *p*-NPS, it is reasonable to consider that one and the same arylsulfatase catalyses the hydrolysis of both substrates in this case. However the possibility of more than one arylsulfatase being present cannot be excluded.

This arylsulfatase is strongly inhibited by phosphate and fluoride but hardly affected by cyanide. So it is very similar to the Type II arylsulfatase of mammalia in this connection. The behaviour of glycosulfatase and myrosulfaminase towards these inhibitors are similar to that of arylsulfatase.

These three sulfatases can be obtained in the 100,000×*g* supernatant and the existence of particulate sulfatase cannot be observed.

SUMMARY

1. Methods of synthesis of potassium *p*-nitrophenyl sulfate and potassium glucose monosulfate were improved.
2. Most part of the original activity of arylsulfate, glycosulfatase and myrosulfaminase of the liver of *Charonia lampas* was found in the 100,000×*g* supernatant fraction.
3. The characteristics of the three enzymes have been investigated. pH optimum are 5.9–6.2 (aryl) and 5.75–5.92 (glyco and myro). NCS activity is competitively inhibited by *p*-NPS, inhibited not competitively by GS and not inhibited by sinigrin. The three enzymes are strongly inhibited by fluoride and iodine, but are hardly affected by cyanide, 8-hydroxyquinoline and EDTA. The three enzymes are competitively inhibited by phosphate.
4. Histological survey of the liver of *Charonia lampas* was carried out.

The author thanks Prof. F. Egami for his interest and encouragement, Dr. T. Sato for the histological survey and Dr. Z. Nagashima for the gift of sinigrin. A part of the expense of this study was defrayed by a grant from the Ministry of Education and from Seikagaku-Kenkyujo Ltd., to which her thanks are due. Some of the experiments were carried out in the Marine Biological Station of Nagoya University.

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ON THE MECHANISM OF THIAMINE ACTION IN THE CARBOXYLASE REACTION

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Many speculations (*1*)–(*20*) have been proposed concerning the action of thiamine as a coenzyme constituent during the course of the thiamine catalyzed enzyme reaction, since the Langenbeck's cycle was first proposed by Langenbeck (*1*). His assumption on the catalytic action of thamine by its 6-amino group of pyrimidine nucleus was rejected by Stern *et al.* (*2*) because of the chemical inertness of this amino group. Lipmann (*3*) observed the colour change during the action of hydrosulfite on thiamine and concluded the hydrogenation of the thiazole ring. Later Zima *et al.* (*4*) suggested another possibility that thiamine might react as disulfide form during its action. But both these hypotheses were later disproved by Karrer (*5*). Acyloin condensation, which is also catalyzed by thiamine-requiring enzyme, was studied by other workers. Ugai *et al.* (*6*) found that the furoin condensation is catalyzed by thiazolium derivatives including thiamine, and this led to the hypothesis proposed by Mizuhara *et al.* (*7–9*) that the acetoin is formed from diacetyl or pyruvate via the active intermediate (Diagram 1, (I)). The formation of acetyl carbanion as an intermediate was also explained by another possible deprotonated form of thiamine (Diagram 1, (II)) (*10*, *11*). But the failure to prove the loss of deuterium from *N*-benzyl-(α-d_2)- 4-methyl thiazolium bromide for catalysis of furoin condensation (*12*) and a small uptake of deuterium into thiamine during its catalytic action (*13*) excluded the possibility of such an intermediate. Another deprotonated form of thiamine (Diagram 1, (III)) was proposed by Breslow (*12*, *14*, *15*) and Downes *et al.* (*16*) in view of the similarity between the benzoin condensation catalyzed by cyanide ion and the acyloin condensation catalyzed by thiamine or thiazolium salt, and such prototropic activity was demonstrated by the rapid deuterium exchange (*14*, *20*).

No experimental approach was made, however, by the enzymic studies except the study of Fry *et al.* (*19*), which appeared shortly before the finish of this work. The purpose of this paper was to examine the deuterium exchange between solvent and coenzyme in the carboxylase reaction suggested by the above cited ylid hypothesis.

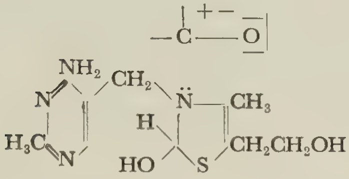
EXPERIMENTAL

Cocarboxylase of 91 per cent purity was kindly supplied by Takeda Pharmaceutical

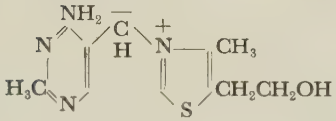
Industries. Thiamine was determined by the thiochrome method. The paper chromatography was carried out on the filter paper Toyo-Roshi No. 53 using solvent described by Mano (*21*), and for spray reagent the mixture of 50 ml. of 55 per cent alcohol, 50 ml. of 10 per cent sodium hydroxide and 61 ml. of 2.5 per cent ferricyanide solution was used. For gradient elution of thiamine with Amberlite IRC 50 XE 64, a column of 20 ml. of H form resin with the diameter of 1 cm. was used and elution was carried out by the use of 1/10 N HCl gradually mixed into 100 ml. of water*. D_2O having 99.8 per cent purity was purchased from Asahi Chemical Industry Co. and from Norsk Hydro Elektrisk. Deuterium analysis was kindly carried out by Dr. Horibe in Tokyo Metropolitan University with Hitachi RMD-1 mass spectrometer.

DIAGRAM 1

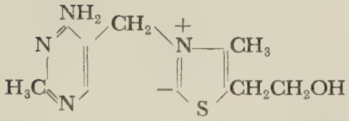
The proposed intermediate in thiamine action



(I) Mizuhara *et al.* (1951) (*7*, *9*)



(II) Wiesner *et al.* (1956) (*10*)
Breslow (1956) (*11*)



(III) Breslow (1957) (*12*, *14*, *15*)
Downes *et al.* (1957) (*16*)

Carboxylase was prepared according to Green *et al.* (*22*) with some modification. One handred grams of dried bakers yeast** was extracted with 300 ml. of $M/2$ phosphate buffer at pH 7.2 for $2^1/_2$ or 3 hours at 37°. After addition of 400 ml. of water to this solution, it was centrifuged and the supernatant was treated with negative absorption by calcium phosphate gel, fractionated with ammonium sulfate and 0.42–0.52 saturated fraction was obtained. The enzyme was then treated with ammoniacal ammonium sulfate solution two times to cleave the coenzyme, washed with saturated ammonium sulfate solution, dialized against 1/200 M citrate buffer pH 6.0 in a cold room for 8 hours to remove the ammonium sulfate almost completely. The enzyme solution was then freeze-dried and immediately used for the experiment. Too long preservation caused denaturation. This enzyme had the Q_{CO_2} of 2800–3600 after alkali treatment. Protein concentration was determined by the method of Warburg and Christian (*23*). Takadiastase, commercially available for determination of thiamine, was used as phosphatase.

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* according to the private communication from J. Suzuoki.

** supplied by Oriental Yeast Industry Co.

RESULTS

Carboxylase reaction was carried out in manometer vessels of Warburg's apparatus at 30°. The contents of the vessel is shown in Table I. The course of the CO_2 evolution is shown in Fig. 1.

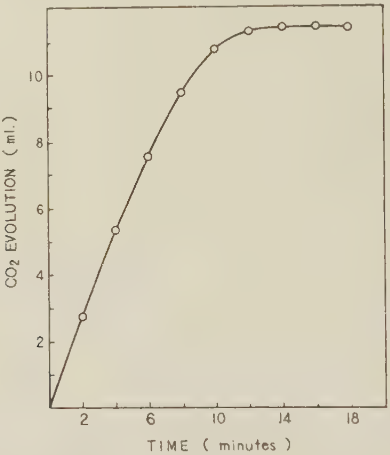

FIG. 1. CO_2 evolution by carboxylase reaction. Conditions are described in Table I. Under these conditions the amount of CO_2 evolved in blank experiment was zero. Temperature: 30°, Gas phase: air.

TABLE I

Contents of Warburg's Manometer Vessel for Carbcxylase Reaction

Main flask:	
thiamine diphosphate,	26.8 mg.
manganese chloride,	21.6 mg.
deuterium oxide,	15 ml.
citric acid,	15 mg.
sodium citrate,	368 mg.
enzyme,	1.3 g.
Side arm:	
sodium pyruvate,	192 mg. in 1 ml. of D_2O

Table I shows the components used for Exp. 1. Exp. 2, Exp. 3 and blank experiment were carried out in half scale. Solid citric acid and sodium citrate were used. The enzyme used for Exp. 3 was obtained from ammonium sulfate fraction of 0.42–0.10 saturation.

After the reaction stopped, pH was adjusted to 4.8 by the addition of hydrochloric acid and 300 mg. of Takadiastase was added to the reaction mixture. After incubation for 44 hours at 37° further 300 mg. of Takadiastase was added to complete its action. After total 70 hours incubation, during which the enzyme became hydrolyzed, the resulting clear solution was deproteinized with perchloric acid, and then neutralized with potassium hydroxide. The cooled supernatant containing about 80 per cent of the added thiamine was extracted four times with its equal volume of water-saturated phenol. To this phenol extract the equal amount of ether was added, the water layer thus obtained was washed with a small portion of ether, and was run onto the column. About 40–50 per cent of thiamine was lost by this step.

After washing the column with 450 ml. of water, thiamine was eluted with 1/10 *N* HCl by gradient elution. Measuring the absorption of the eluted solution at 247 mμ, only a single peak appeared. The eluates of this fraction were combined and examined with paper chromatography, which showed the only one substance having the same R_f value as with the authentic thiamine, except a trace of fluorescent substance. To 10 ml. of this solution containing 2.1 mg. of thiamine, 28.2 mg. of carrier thiamine was added, and thiamine reineckate was prepared, recrystallized, and dried over P_2O_5. It decomposes at146–150°, the same temperature as authentic thiamine reineckate. It was then combusted under oxygen to get water which was reduced to hydrogen with zinc and its deuterium content was analysed. The results are shown in Table II.

TABLE II

Deuterium Contents of Thiamine Reineckate obtained after the Carboxylase Reaction

Exp. No.	Enzyme	Q_{CO_2}	Cocarboxylase added	Thiamine dilution	D incorporated	Atom of D calculated
	(*g*)		(*mg.*)	(*times*)	(%)	(*atom*)
1	1.3	3600	26.8	14.4	0.486	0.146
2	0.6	2800	13.56	12.6	0.270	0.081
3	0.7	3500	13.2	17.0	0.316	0.095
blank			11.9	11.4	0.188	0.056

The column next to the last indicates the values for deuterium contents of the thiamine reineckate, corrected for the dilution factor. Figures in the last column indicate the calculated rates of D atom to the hypothetically exchangeable one hydrogen atom in one molecule of thiamine.

DISCUSSION

The values in the last column in Table II indicate a small exchange of hydrogen atom in one molecule of thiamine, which must contain one or two deuterium atom according to the ylid hypothesis. This calculation was, however, based on the assumption, that all the coenzyme added to the reaction mixture participates in the reaction, during the course of the decarboxylation of pyruvate. Although the validity of this assumption can not be determined, the increased requirement of coenzyme for maximum carboxylase activity after alkali treatment as reported by Green *et al.* (*22*) shows much more participation of coenzyme than that contained in the original holoenzyme before alkali treatment. The gradual saturation curve of apoenzyme against coenzyme suggests the equilibrium state of enzyme-coenzyme dissociation. The amounts of carbon dioxide evolved was 10 times larger than the values calulated from an assumption that one mole of cocarboxylase added gives rise to the evolution of one mole of carbon dioxide.

Carrier thiamine was added only after the thiamine was isolated from the reaction mixture, because of the fear that the thiamine of the cocarboxylase bound to the enzyme might not be isolated by the separation procedure suitable for free cocarboxylase. Takadiastase which liberates the thiamine quantitatively from the tissue was used for the separation of thiamine from the reaction mixture. The significant loss as much as 40–50 per cent which happened on the phenol extract step is also the case with free thiamine. Care was taken to minimize the eventual loss of deuterium during the isolation procedure, by keeping the pH of the solution always acidic during the whole operation. Deuterium possibly incorporated via the intermediate III, should be washed out under these isolation procedures because of the high prototropic activity of thiazole 2-C. Cleavage by bisulfite was not attempted because it was not sure whether this procedure causes the loss of deuterium or not, when this work was started.

SUMMARY

1. The validity of the ylid hypothesis on the catalytic action of thiamine in carboxylase reaction was examined.

2. Cocarboxylase was added to apocarboxylase in the mediume of D_2O and recovered as thiamine after the enzymic reaction was carried out using pyruvate as substrate.

3. The thiamine reineckate obtained was analysed for deuterium contents.

4. These values are too small to follow the ylid hypothesis under the assumption that the reversible dissociation of enzyme-coenzyme complex exists.

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STUDIES ON HEMOGLOBIN

III. ARGININE-PEPTIDES IN THE TRYPTIC AND CHYMOTRYPTIC HYDROLYSATES OF BOVINE AND HORSE GLOBINS

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The molecular weight of vertebrate hemoglobin appears to fall usually in the neighborhood of $65\sim70\times10^3$, although under some conditions the weight is about half of this, indicating that the protein dissociates into two halves (*1*, *2*, 3). In the previous communications on the N-terminal amino acid sequence of a variety of hemoglobins (*4*), and also that of the arginine-peptides in the *Streptomyces* proteinase digest of horse and bovine hemoglobins (*5*), we pointed out that several amino acid sequences in the molecules occurred in a concentration of two moles per 68×10^3 g. These findings seemed to be further evidences for the existence of hemoglobin as a dimer, consisting of two similar monomers.

Recently, however, there have been reports which indicate the existence of four subunits (*3*) of two different kinds (*6*) in hemoglobin molecule. The presence of three different pairs of N-terminal sequences, [Val-Leu-]$_2$ [Val-Glu-]$_2$ [Val-Gly-]$_2$, in horse hemoglobin (*4*) complicates the consideration of the dissociation into four subunits ((*3*), see also (*7*)), differing from the other types of hemoglobin (bovine, human or snake) which are composed of two different N-terminal pairs, [Val-Leu-]$_2$ [Val***-Gly]$_2$ (*4*). The horse hemoglobin half molecules might consist of only two fundamental and common major types of chains, Val-Leu ····· and Val-Gly ·····, one of which is branched from a ε-amino group of lysine residues (*3*) and terminated at the Val-Gly- sequence. Alternatively it might consist of three isolated peptide chains with the third being derived from the splitting of a definite peptide bond in one of the two major chains, ·····$\downarrow$Val-Glu ·····, and the third might have escaped from the detection by the method employed.

But there have been so few reports (*4*, *5*) on the amino acid sequences in hemoglobin that it is difficult to compare the degree of differences between the two types of hemoglobins, two- and three-N-terminal group types. It is pertinent to note that all fourteen arginine residues in horse and bovine hemoglobin were found to be present in pairs of seven different sequences and these sequences in the two hemoglobins were very similar.

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*** The valyl residue is replaced with methionyl in the case of bovine hemoglobin (*4*).

EXPERIMENTS

Materials—Horse and *bovine globins* used in this experiments, were the same preparation as used in the previous paper (*5*). The both air-dried samples had 15.0±0.1 per cent of N (Kjeldahl), 12±0.5 per cent of moisture and 14 μM of arginine per 75 mg. (corresponds to 1 μM, 68 gm. of moisture free hemoglobin) (*5*). A commercial preparation "Trypsilin" from Mochida Seiyaku Co. was used as the *trypsin* source. It was contaminated with a few per cent of chymotrypsin. Crystalline chymotrypsinogen was obtained from a $N/10$ sulfuric acid extract of beef pancreas according to the method reported by Kunitz and Northrop (*8*).

Methods—Hydrolysis: Eight hundred mg. of globin were dissolved in 50 ml. of $M/20$ sodium phosphate buffer (pH 7.0) and heated in a boiling water bath for 15 minutes to denatur the protein. The suspension of the denatured protein along with 8 mg. of trypsin was incubated at 37° for a given time (Tryptic hydrolysate). To the fifteen hour trypsin hydrolysate, 8 mg. of chymotrypsinogen was added and the suspension was incubated at 37° for additional 15 hours (Tryptic chymotryptic hydrolysate). The degree of hydrolysis was conventionally followed by the increment of Sakaguchi-reaction color density (*9*) in the supernatant of an aliquot of the reaction mixture, after the addition of an equal volume of 10 per cent trichloroacetic acid. The increment was expressed in terms of the arginine concentration which gave the same color intensity.

Chromatographic Separation of Arginine-Peptides—The hydrolysate mixture corresponding to 10 μM of hemoglobin, was evaporated to dryness under reduced pressure and the residue, suspended in 1 ml. of distilled water, was added to a column of Amberlite IRC-50 (1.2×60 cm.), previously equilibrated with $M/10$ sodium citrate buffer (pH 5.4) (*10*). The column was then eluted with sodium chloride in the same buffer (NaCl 6.8 g., citric acid monohydrate 10.5 g. and sodium hydroxide 5.0 g. in 500 ml. of water. pH 5.4 Na^+. 0.48 g. ion per liter) and finally with N sodium hydroxide. All effluents were collected by an automatic fraction collector and analysed as follows:

Spectrophotometric Determination of Arginine-Peptides—The concentration of arginine-peptides was assayed spectrophotometrically by the Sakaguchi-reaction, using arginine as the standard (*5*, *9*). The peptides, examined here, gave the same color intensity both before and after the hydrolysis within a range of 5 per cent (*5*).

Purification of Arginine-Peptides—The fractions corresponding to each Sakaguchi-reaction positive peak on the elution diagrams, were purified by passing through a column of Amberlite IRA-411 (OH) (3×15 cm.) to remove the contaminating non-arginine peptides as reported previously (*5*). The arginine peptides in the filtrate, and the washings of the resin column (100 ml.), were added to a column of Amberlite IR-112 (H) (3×15 cm.).

Each adsorbed peptide, after washing with 50 ml. of water, was eluted with a small amount of 2 N ammonia to remove the greater part of the contaminating salts. The rate of flow was adjusted to about 1 ml. per minute throughout these treatments. The ammonia eluate was concentrated to dryness under reduced pressure and the residue was used for the following analysis.

Paper Chromatography of Arginine-Peptides—The samples containing to 0.1∼0.5 μM of arginine were chromatographed on Toyo filter paper No. 51, using *n*-butanol : acetic acid :

water (5 : 2 : 2 v/v) as the solvent system and reacted with both ninhydrin and Sakaguchi-reagent (*11*, *12*) to determine the contamination of non-arginine peptides (*5*).

Amino Acid Composition of Arginine-Peptides—Samples of the purified arginine-peptides (about 1 mg.) were heated with 1 ml. of 6 *N* HCl at 115° for 8 hours, and the resultant amino acids were identified by paper chromatography (*12*). Their amounts were estimated semiquantitatively by comparing the ninhydrin-colored spot area with those of known quantities (*12*, *13*). In the case of arginine-peptide G, which was diazo-reaction positive but Millon-reaction negative, the ratio of the color densities in the Sakaguchi (*9*) and Pauly diazo reactions (*14*) was measured to estimate the molar ratio of arginine and histidine residues. The absence of tryptophan residues in each arginine-peptide was verified by the absence of ultra-violet absorption at 280 mμ.

N-Terminal Groups of Arginine-Peptides—Sample of the purified arginine-peptides (about 3 mg.) were dinitrophenylated and the DNP-derivatives, after fractionation by ether extraction and passage through talc-column (*5*, *15*), was heated with 1 ml. of 6 *N* HCl at 100° for 2～5 hours. The resultant DNP-amino acids and free amino acids were identified by paper chromatography. Both *n*-butanol saturated with 2 *N* ammonia and 1.5 *M* phosphate buffer (pH 6) were used as the solvent system for DNP-amino acids (*16*).

Another aliquot of DNP-arginine peptide was used for the determination of the ratio of absorption at 360 mμ (in 4 per cent $NaHCO_3$) and Sakaguchi-reaction color density, to estimate the ratio of amino- and guanidino-groups in the molecule (*5*).

RESULTS

Tryptic Hydrolysis of Bovine Globin—By the action of trypsin on the heat denatured bovine globin, about 40 per cent of the arginine residues in the protein was very rapidly (within 30 minutes) and the remaining 50 per cent was rather gradually (within 10 hours) converted into a fraction soluble in 5 per cent trichloroacetic acid, as shown in Fig. 1. Throughout the hydrolysis, appreciable amounts of insoluble precipitate were present and more than 25 per cent of total N remained as such insoluble tryptic core, even after 30 hours. The core, however, contained only minute amount of arginine (less than 20 per cent of the total arginine).

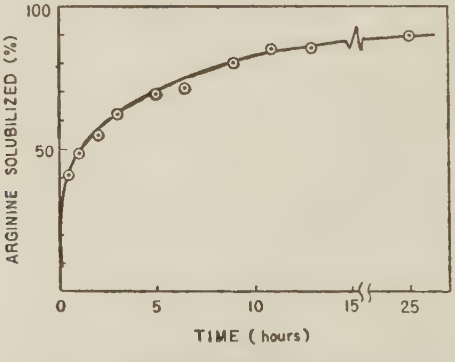

FIG. 1. Tryptic hydrolysis of globin.

From the 30 hours-hydrolysate, seven arginine-containing fractions A, B, C, D, E, F and G, were separated on a column of Amberlite IRC-50 (pH 5.4) as shown in Fig. 2 (curve –○–). Whereas the 5 hours-hydrolysate, though more than two third of the total arginine was already converted into a trichloroacetic acid-soluble fraction, gave fraction C as the only major component (1.3 μM per μM protein) on the elution diagram. The quantities of these arginine-fractions estimated from the elution diagram, are summarized in Table I. The sum of these quantities together with several minor components between peaks B and C, corresponded to about 20 and 60 per cent respectively of the total arginine in the 5 and 30 hours trypic hydrolysate added to the column, and the remaining ones could not be eluted from the column even by 1 *N* sodium hydroxide (*23*). The peptides A, B, C, D, E, F and G, however, were almost quantitatively recovered from a column of the rechromatography under the same conditions. These findings suggest that there were appreciable amounts of other larger peptides in the tryptic hydrolysate. The fractions corresponding to each peak, shown as A~G in Fig. 2, gave only one spot on paper chromatogram colored by the Sakaguchi-reaction, R_f values of which are shown in Table I. On the paper chromatogram colored by ninhydrin, however, several other spots were observed in all cases, together with one main spot which was also Sakaguchi-reaction positive. These contaminating non-arginine peptides (ninhydrin positive, Sakaguchi-reaction negative spots) were easily removed from the fractions C, D, E, F and G, by pass through a

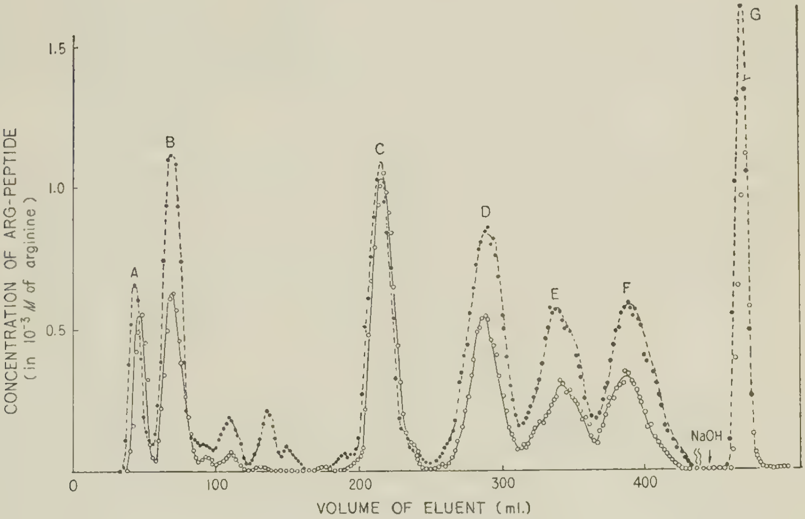

FIG. 2. Elution diagrams of Arginine-peptides from bovine globin.
—— : 30 hours-tryptic hydrolysate
- - - - : Tryptic and chymotryptic hydrolysate

column of Amberlite IRA-411 (OH) and subsequent fractional elution from a Amberlite IR-112(H) with 2 N ammonia. The loss of arginine peptides was less than 20 per cent during this procedure. Whereas the purifications of arginine-peptides A and B were only achieved with the twice or three times repeated treatment with a column of Amberlite IRA-411 (OH) and with about 30~50 per cent loss of the peptides.

The amino acid constituents of these purified arginine-peptides and of the DNP-derivatives are shown in Table I. These DNP-arginine peptides did not contain lysine residue and gave the same ratio of absorption at 360 mμ due to amino-N-DNP-group and Sakaguchi-reaction color density due to arginine residue as those of DNP-arginine within a range of 5 per cent. The fact indicates that they were free from other non-arginine peptides and had only one arginine residue in the molecule.

Tryptic and Chymotryptic Hydrolysate of Bovine Globin—To hydrolyse the above-mentioned larger peptides, which could not be eluted from a column of Amberlite IRC-50, chymotrypsinogen was added to the fifteen hours-trypsin hydrolysate and the mixture was further incubated for fifteen hours. By the action of chymotrypsin, which was rapidly produced from chymotrypsinogen (under these conditions), the trypsin core of globin dissolved rapidly into the solution and the recovery of arginine residues from a column of Amberlite IRC-50 reached as much as 90 per cent. As shown in Fig. 2 (curve ··●··) the concentration of fractions B, D, E, F and G, increased to about 2 (1.6~2.2) moles per mole of hemoglobin, whereas those of fraction A still remained as low as 0.6 mole. The concentration of fraction C was already as high as 2 moles by the action of trypsin only and did not change by the additional action of chymotrypsin.

The positions of these seven main fractions on the elution diagram and their R_f values on the paper chromatogram were just the same as these from the tryptic hydrolysate within the experimental error. The identity of these seven arginine peptides in the both hydrolysates, was further verified by the analysis of the amino acid composition and of the N-terminal groups. From these analysis, it was also confirmed that peptide D (Ala-Arg) was contaminated with small amount of free arginine and fraction G contained His-Arg together with Ala-His-His-Arg, in the case of tryptic and chymotryptic hydrolysate, respectively. These results are summarized in Table I.

Tryptic Hydrolysis of Horse Globin—There were no remarkable differences in the time courses of tryptic hydrolysis between horse and bovine globins. And seven arginine-fractions were also separated on a column of Amberlite IRC-50 from the 30 hours tryptic hydrolysate of heat-denatured horse globin. Their yields, amino acid compositions and N-terminal groups were just the same as those in the case of bovine, globin, except for fractions B and G. Fraction G was mainly composed of His-Arg in this case, though trace amount of alanine could be detected as the N-terminal group. Peptide B, which behaved chromatographically pure after the repeated treatment of Amberlite IRA-411 (OH) column, gave arginine and glutamic acid together

TABLE I

Arginine-Peptide from Bovine Globin

No.	Yield (μM/75 mg.)[a]			R_f	Amino acid composition	
	5-Hrs-Try	30-Hrs-Try	15-Hrs-Try then 15-Hrs-Chy		Before DNP-ation	After DNP-ation
A	0.05	0.5	0.6	0.21	Arg,Asp,Val	DNP-Val,Arg,Asp
B	0.05	0.9	1.6	0.33	Arg,Glu,Leu,Val	DNP-Glu,Arg,Leu,Val
C	1.30	2.0	2.0	0.20	Arg,Glu,Thr	DNP-Thr,Arg,Glu
D	0.10	1.2	2.2	0.25 (0.29)[b]	Ala,Arg	DNP-Ala,Arg[c]
E	0.07	0.8	1.8	0.19	Arg,Gly	DNP-Gly Arg Dinitrophenol[d]
F	0.08	1.0	2.1	0.54	Arg,Leu	DNP-Leu Arg
G	0.10	1.3	1.7	0.15	Ala,Arg,$His_{1.9}$[e]	DNP-Ala, Arg, Dinitroaniline[f]

a) 75 mg. of air-dried sample corresponded to 1 μmole of hemoglobin (68 mg.). Try and Chy show tryptic and chymotryptic hydrolysis for a given time, respectively.

b) In the case of 15-Hrs-Try-15-Hrs-Chy, minor component (free Arg) was also observed.

c) In the case of 15-Hrs-Try-15-Hrs-Chy, small amount of DNP-Arg was also observed.

d) Degradation product of DNP-Gly.

e) In the case of 15-Hrs-Try-15-Hrs-Chy, the ratio of His and Arg was 1.4.

f) Degradation product of imidazol-DNP-His.

TABLE II

Arginine-Peptide from Horse Globin

No.	Yield (μM/75 mg.)[a] 30-Hrs-Try	R_f	Amino acid composition	
			Before DNP-ation	After DNP-ation
A	0.6	0.23	Arg,Asp,Val	—
B	1.0	0.31	Arg,Glu,others	? Arg,Glu
C	1.8	0.19	Arg,Glu,Thr	DNP-Thr,Arg,Glu
D	1.2	0.24	Ala,Arg	DNP-Ala,Arg,
E	1.1	0.19	Arg,Gly	DNP-Gly,Arg,Dinitrophenol[b]
F	0.8	0.54	Arg,Leu	DNP-Leu,Arg
G	1.0	0.13	Arg,$His_{1.1}$(Ala)	Dinitroaniline[c] Arg, (DNP-Ala)

a) 75 mg. of air-dried sample corresponded to 1 μmole of hemoglobin (68 mg.). Try shows tryptic hydrolysis for a given time.

b) Degradation product of DNP-Gly.

c) Degradation product of imidazol-DNP-His.

with less amounts of several other amino acids* by the acid hydrolysis. The fact indicates that fraction B was a mixture of several similar peptides [X, —Glu]-Arg. These results are summarized in Table II.

DISCUSSION

From the above-mentioned experimental results and the well-known substrate specificity of trypsin (*17–20*) and chymotrypsin (*17*, *21*, *22*), it could be concluded that bovine hemoglobin had the following amino acid sequences and that most of them (B∼G) are present in pairs in the molecule.

A X-Val-Asp-Arg ↑ Y   B ϕ ↓ Glu[Leu,Val]Arg ↑
C Ba ↑ Thr-Glu-Arg ↑   D ϕ ↓ Ala-Arg ↑
E ϕ ↓ Gly-Arg ↑   F ϕ ↓ Leu-Arg ↑
G ϕ ↓ Ala-His ↓ His ↓ Arg ↑

↑↑ : Hydrolysis by trypsin
↓↓ : Hydrolysis by chymotrypsin
Ba : Lys or Arg   ϕ : Aromatic amino acid and so.

In the previous paper (*5*), one of the authors (Satake) has already elucidated that bovine hemoglobin has fourteen arginine residues in the molecule, six of which are present as pairs of Ala-Arg, Gly-Arg and Arg-Glu. So one of the arginine residues of peptides A, B, C, F, or G must be followed by glutamyl residues in the hemoglobin. The N-terminal groups of peptides B, D, E, F and G might have been attached with one carboxyl group of aromatic amino acids and so, whereas that of peptide C with those of lysine or arginine residues in the original protein. Because peptide C was the only one, rapidly produced by the action of trypsin and the other six were liberated by the additional action of chymotrypsin. The production of His-Arg and free arginine by the prolonged action of chymotrypsin on the tryptic hydrolysate, may be due to the slow hydrolysis of carboxyl site of histidyl residues of Ala-His-His-Arg, by the chymotrypsin. The action of chymotrypsin in such a manner has been reported, also by Hirs (*22*) in the case of oxidized ribonuclease. The species difference between His-Arg from horse hemoglobin and Ala-His-His-Arg from bovine, may be due to the different resistance of -His-His- bond to chymotryptic hydrolysis. The low yield of peptide A may be due to Trypsin resistance of Arg-Y bond (for example, Y is Pro (*22*, *26*)), or due to the resistance of x-Val bond to tryptic and chymotryptic hydrolysis.

The present findings that the most arginine residues occurred in pairs of the sequences in hemoglobin, seem to be another support for the existence of the protein as a dimer composed of similar subunits, which has been proposed from the change of the molecular weight during the urea-, acid- or surface-denaturation (*1–3*), from the X-ray analysis of the crystal (*24*) and from the sulfhydryl analysis (*25*). The present results are also by any means inconsistent with the assumption that hemoglobin is a sort of tetrad myoglobin molecule ((*3*) see also (*7*)). Because some evidences for the existence of two different subunits in the molecule has been obtained by Reichmann and Colvin (*6*), who studied the molecular weight and the electrophoretical separation of performic acid-oxidized globin, and by the present authors who studied the N-terminal amino acid sequences (*4*), a hemoglobin might be concluded to be composed of a pair of two different subunits.

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* Mainly leucine, valine and alanine. In some experiments, only former two were identified.

It will be very interesting, that horse hemoglobin had the similar arginine peptide sequences as those of bovine hemoglobin, inspite of the great difference in N-terminal sequences (*4*). The result, however, seems to be far from the evidence to decide whether or not the additional N-terminal sequence, Val-Glu- in horse hemoglobin is derived from the splitting of a definite bond in one of two major chains, as mentioned in the introduction part.

SUMMARY

Bovine hemoglobin, which contained 14 arginine residues per 68×10^3 *g*, had the following seven arginine-peptide sequences in the molecule:

A Val-Asp-Arg,
B Glu [Val,Leu] Arg
C Thr-Glu-Arg
D Ala-Arg
E Gly-Arg
F Leu-Arg
G Ala-His-His-Arg

and their concentrations were found to be 2 *M* per 68×10^3 g, except for Peptide A.

Horse hemoglobin also had a very similar arginine peptide sequences inspite of the great difference in N-terminal sequences.

These results suggest that hemoglobin is a dimer consisting of two similar (or same) monomers.

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CHROMATOGRAPHIC SEPARATION OF DNP-AMINO ACIDS

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(Received for publication, August 17, 1959)

Chromatographic separation of *N*-2,4-dinitrophenyl-amino acid (DNP-amino acid) was first carried out by F. Sanger using a silica gel column (*1*). Since then various chromatographic methods have been published, of which the buffered column of J. L. Perrone (*2*) and the paper chromatographic method of A. L. Levy (*3*) and of G. Koch and W. Weidel (*4*) are most widely used.

It has also been shown that aromatic substances are strongly adsorbed by ion exchange resins (*5–13*), and phenol and substituted phenols can be separated chromatographically on columns of cation exchange resins (*14*, *15*). This finding prompted the author to investigate the separation of DNP-amino acids on cation exchange resins. The separation of the common DNP-amino acids with these resins has now been achieved.

Carboxylic acid type resin Amberlite IRC-50 and sulfonic acid type resin Duolite C-25* were used as adsorbents. Since the p*K* of the DNP-amino acids is lower than that of the fatty acids, it was necessary to use acidic solvents to suppress the ionization of the carboxyl group in both solute and resin. The mixture of DNP-amino acids was first separated on columns of Amberlite IRC-50 and the fractions containing more than one DNP-amino acids were combined and separated on a column of Amberlite IRC-50 or Duolite C-25. Ethyl acetate insoluble DNP-amino acids were separated on a column of Duolite C-25.

EXPERIMENTAL

DPN-Amino Acid—DNP-amino acids were synthesized by the method of A. L. Levy and D. Chung (*18*). DNP-L-Leucine was kindly supplied by Mr. T. Matsushima. ε-DNP-lysine was kindly supplied by Dr. T. Ikenaka. A sample of DL-*allo* threonine was made available by Dr. Y. Izumi. DL-*allo* Isoleucine mixed with some DL-isoleucine was kindly supplied by Mr. T. Inui.

Adsorbent—Amberlite IRC-50 (A. G.) was pulverized and washed as previously described

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* In an earlier stage of this investigation, Amberlite IR-112 was used (*16*). Since this resin is now obsolete, I substituted Duolite C-25, a sulfonic acid type cation exchanger with an amorphous polystyrene matrix (*17*).

(200–300 mesh) (*19*). Duolite C-25 was pulverized and screened as described (200–300 mesh) (*20*) and washed with ten volumes of 6 *N* hydrochloric acid and 2 *N* sodium hydroxide (*21*). Both resins were used in the hydrogen ion form.

Chromatographic Column—Details of the chromatographic tube were described previously (*19*). The ion exchange resin suspended in the solvent to be used for chromatography was poured through a funnel into the chromatographic tube and allowed to settle. The sizes of the columns are indicated in Table I.

Automatic Fraction Collector—A siphon type automatic fraction collector was used without a cotton plug at the bottom of the collecting cup* (*22*). The volume of the siphons

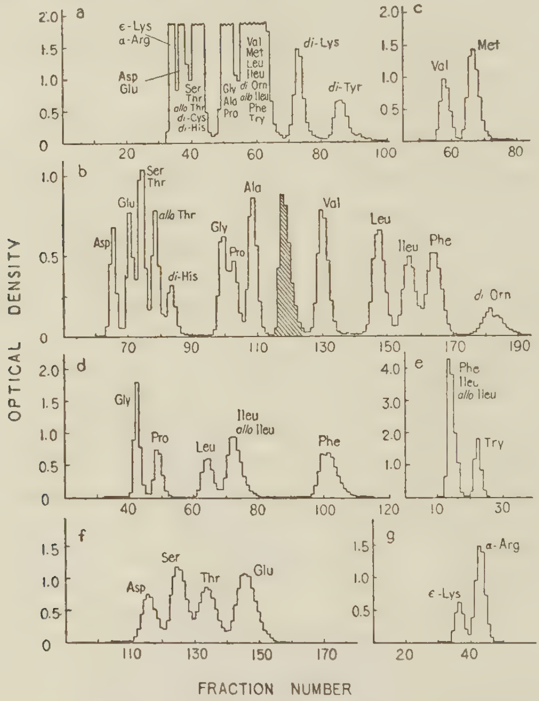

FIG. 1. Elution pattern of DNP-amino acids. Shaded peak: dinitrophenol. DNP-is omitted in the figure.

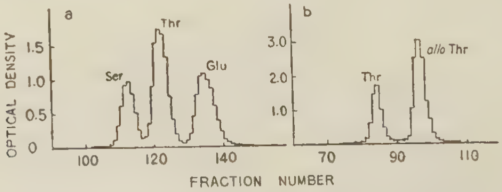

FIG. 2. Elution pattern of DNP-amino acids. DNP- is omitted in the figure.

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* The inner diameter of the capillary tube connecting the collecting cup and the siphon was 2 mm.

were 1.0 ml. and 2.0 ml.

Chromatographic Separation of Synthetic Mixtures—DNP-Amino acids were dissolved in the solvent to be used for chromatography and 1.0 ml. of the solution was added to the column. When the DNP-amino acids did not dissolve readily in the solvent to be used, they were first dissolved in the organic component of the solvent and then 0.2 *N* or 0.3 *N* hydrochloric acid was added until the ratio of organic component to dilute hydrochloric

TABLE I

Composition of the Solvents

Solvent	Composition (v/v)	
A	MEK1)-Me_2CO2)-0.3 *N* HCl	1 : 3 : 4
B	MEK-Me_2CO-0.3 *N* HCl	1 : 3 : 5
C	MEK-Me_2CO-0.3 *N* HCl	2 : 1 : 9
D	MEK-Me_2CO-0.2 *N* HCl	2 : 1 : 6
E	AcOH3)-0.3 *N* HCl	1 : 5
F	AcOH-0.3 *N* HCl	1 : 2
G	MEK-3 *N* HCl	1 : 3
H	MEK-0.2 *N* HCl	2 : 7

1) MEK : Methylethylketone.
2) Me_2CO : Acetone.
3) AcOH : Glacial acetic acid.

TABLE II

Conditions of the Chromatographic Separation

Figure	Resin	Size of the column (*cm.*)	Solvent	Room temperature (°*C*)	Volume of one fraction (*ml.*)
1-a	Amberlite IRC-50	1.0 ×60	A	20	1
1-b	Amberlite IRC-50	0.9 ×118	B	13–15	1
1-c	Duolite C-25	0.8 ×97	E	15–20	2
1-d	Duolite C-25	0.8 ×97	E	15–20	2
1-e	Duolite C-25	0.8 ×55	F	20–23	2
1-f	Amberlite IRC-50	0.76×112	C	25	1
1-g	Duolite C-25	0.76×34	G	20–23	2
2-a	Amberlite IRC-50	0.76×73	H	25–28	1
2-b	Amberlite IRC-50	0.76×88	D	10	1

acid became the same or slightly less than that of the solvent. The effluent was collected in a dark room in 1.0 ml. or 2.0 ml. fractions in test tubes with a graduation mark at 3.5 ml. Quantitative analysis were performed spectrophotometrically. The solvent used

TABLE III

Per Cent Recovery of DNP-Amino Acids from the Chromatographic Column

DNP-Amino acid	Solvent							
	A	B	C	D	E	F	G	H
DNP-Asp	—	102	90	—	—	—	—	—
DNP-Glu	—	103	88	—	—	—	—	87
DNP-Ser	—	98	91	—	—	—	—	100
DNP-Thr	—	100	97	97	—	—	—	95
DNP-*allo*Thr	—	103	—	96	—	—	—	—
DNP-Gly	—	99	—	—	90	—	—	—
DNP-Pro	—	102	—	—	92	—	—	—
DEP-Ala	—	99	—	—	—	—	—	—
di-DNP-Cys	—	94	—	—	—	—	—	—
DNP-Val	—	98	—	—	94	—	—	—
DNP-Met	—	103	—	—	93	—	—	—
DNP-Leu	—	100	—	—	87	—	—	—
DNP-Ileu	—	96	—	—	89	—	—	—
DNP-*allo*Ileu	—	94	—	—	89	—	—	—
DNP-Phe	—	96	—	—	80	—	—	—
DNP-Try	—	—	—	—	—	92	—	—
di-DNP-Orn	—	60–80	—	—	—	—	—	—
di-DNP-Lys	92	—	—	—	—	—	—	—
di-DNP-Tyr	80	—	—	—	—	—	—	—
di-DNP-His	—	102	—	—	—	—	—	—
α-DNP-Arg	—	—	—	—	—	—	102	—
ε-DNP-Lys	—	—	—	—	—	—	94	—

for the chromatographic separation was added to each fraction as a diluent to give 3.5 ml. and the absorbance of each fraction was measured at 350 mμ using a Beckman model DU quartz spectrophotometer.

RESULTS AND DISCUSSION

As shown in Fig. 1, the DNP-amino acids can be separated systematically using six columns. They were first separated on a column of Amberlite IRC 50 yielding di-DNP-Lys and di-DNP-Tyr as separate peaks (Fig. 1-a). The other acids were again separated on a column of the same resin using a solvent mixture containing less of the organic component. In this elution pattern (Fig. 1-b), DNP-Thr overlapped with DNP-Ser, DNP-Met with DNP-Val and DNP-Leu with DNP-*allo*Ileu. Di-DNP-Cys was eluted between

DNP-Ala and dinitrophenol (at 15°) and overlapped with the latter at lower temperatures. When DNP-Try was present, it was eluted with DNP-Leu (at 15°). These DNP-amino acids were separated from each other by another column of Amberlite IRC-50 and the columns of Duolite C-25. The separation of DNP-Ser, DNP-Thr and DNP-Glu could be improved by using solvent H (Fig. 2-a), although in this case DNP-Asp and DNP-Ser were eluted together. For a determination of the diastereoisomers of synthetic threonine solvent D could be used conveniently.

Ethyl acetate insoluble DNP-amino acids were separated using a column of Duolite C-25 with solvent G.

The elution pattern was highly sensitive to changes of the ambient temperature and the range of the optimum temperature was narrow for columns used for the separations represented in Figs. 1b, 1f and 2a. The former had to be used between 10° and 15°, the latter two between 25° and 28°. The capacity of columns was high and the optimum load was 100 to 200 μg. per one component. The recovery was satisfactory with the exception of di-DNP-Orn. The column could be used repeatedly, because the resins were used with acidic solvents. When carefully screened resins were used the flow rate of the solvents did not change even after repeated uses. The flow rate was two to three ml. per hour.

Since the chromatographic method described utilizes non-ionic adsorption of non-charged organic molecules on ion exchange resins, the structure of the resins seems to play an important role in the efficiency of the separation, for example, as in the cases of the separation of DNP-Pro from DNP-Gly, DNP-Met from DNP-Val and DNP-Leu from DNP-*allo*Ileu (Figs. 1-b, c and d). The efficient separation of DNP-Phe from DNP-Ileu (Fig. 1-d) and DNP-Try from DNP-Phe and DNP-Ileu (Fig. 1-e) by the column of Duolite C-25 may be accounted for by its higher content of benzene nuclei than Amberlite IRC-50.

The freedom of selection of the organic component of the solvents is very limited, because alcoholic solvent can not be used because of the danger of esterification of the carboxyl group in both solute and adsorbent. Therefore a mixture of a water miscible ketone (acetone+methylethylketone) or glacial acetic acid was used. By varying the ratio of these solvents some modification of the elution pattern was possible (Figs. 1-f and 2-a).

SUMMARY

The chromatographic separation of the DNP-amino acids was achieved using Amberlite IRC-50 and Duolite C-25 as the adsorbent and dilute hydrochloric acid containing varying amounts of acetic acid or acetone and methylethylketone as the eluent. It was shown that this procedure successfully separated the DNP-amino acids with a recovery of 80 to 100 per cent with the exception of di-DNP-Orn (60 to 80 per cent). Of practical importance was the high capacity of columns (about 500 μg./cm^2/one component) and the ability to separate DNP-*allo*Thr and DNP-*allo*Ileu from other DNP-

amino acids.

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The Journal of Biochemistry, Vol. 47, No. 2, 1960

CHEMICAL STRUCTURE OF "FOURTH FLAVIN COMPOUND"

During the examination of the temperature for extraction of flavins from animal tissues, Yagi (*1*), one of the authors, found that the homogenate of pig liver heated above 90° gives "fourth flavin compound" (FFC) besides ordinary three flavin compounds in the liver, *i.e.*, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and free riboflavin (FR). FFC was also found to be different from lyxoflavin (*2*), flavinyl glucoside (*3*), or FAD-X (*4*) by paper chromatography. Using an aqueous solution of crude FAD, it was found that FFC was produced quantitatively from FAD, and was converted to FMN by heating it in acidic medium.

In this communication, the chemical structure of FFC studied with its crystallized sample (*5*) is briefly reported.

Isoalloxazine nucleus of FFC was determined by lumiflavin fluorescence method (*6*), and phosphoric acid of FFC was determined by the method of Fiske-Subbarow (*7*). From these analyses, it was found that FFC gives equimoles of phosphoric acid and FR. When FFC was hydrolysed by N HCl at 100° for 7 minutes, adenosine derivatives were not found in the hydrolysate by paper chromatography using *n*-butanol : acetic acid : water (4 : 1 : 5, *v/v*) as the mobile phase. The results of elemental analysis also showed that FFC consisted of equimoles of FR and phosphoric acid.

Considering the fact that FMN was produced from FFC by acid hydrolysis, the cyclic compound of phoshoric acid was supposed.

To test this point, the second phosphate dissociation of FFC was examined by paper electrophoresis (*8*). The results showed that FFC has no second phosphate dissociation, while FMN has it.

From these data, the following possible chemical structures of FFC were supposed: riboflavin-4′,5′-cyclic phosphate (I), riboflavin-3′,5′-cyclic phosphate (II), riboflavin-2′,5′-cyclic phosphate (III), and diriboflavin pyrophosphate (IV).

To identify FFC with one of the above four possible structures, consumption of periodic acid by FFC was measured. By these possible substances, periodic acid is expected to be consumed per mole of isoalloxazine nucleus as follows: one mole by (I) or (III), zero mole by (II), and two moles by (IV).

Two ml. of the aqueous solution containing 0.005 M of FFC and 0.02 M of sodium meta-periodate were kept at 0° and pH 5.5. The consumed periodate was estimated by the method of Malaprade (*9*). As shown in Fig. 1, one mole of FFC calculated on the basis of isoalloxazine nucleus did not consume more than 1.13 moles of periodate after 72 hours. From these results, the possible structure was considered to be (I) or (III).

As a standard test, the consumptions of periodate by FR and FMN were estimated, and they consumed two and three moles of periodate respectively as theoretically expected.

The decomposition products of three flavin compounds by periodate oxidation were then analyzed by paper chromatography using 5 per cent aqueous disodium hydrogen phosphate. They were identified each other, having the same R_f value of 0.13.

This decomposition product was obtained as yellowish orange crystal of needle form. It reduced both Tollens' silver reagent and Fehling solution. This compound was converted easily to lumiflavin by the photodecomposition in alkaline medium, and to lumichrome by that in acidic medium. By oxidizing this compound with bromine water, it was converted to another substance, which was identified with 6,7-dimethyl isoalloxazine-9-acetic acid (*10*) by paper chromatography. ($R_f=0.45$ by 5 per cent disodium hydrogen phosphate, and $R_f=0.34$ by *n*-butanol : acetic acid : water (4 : 1 : 5, *v*/*v*)).

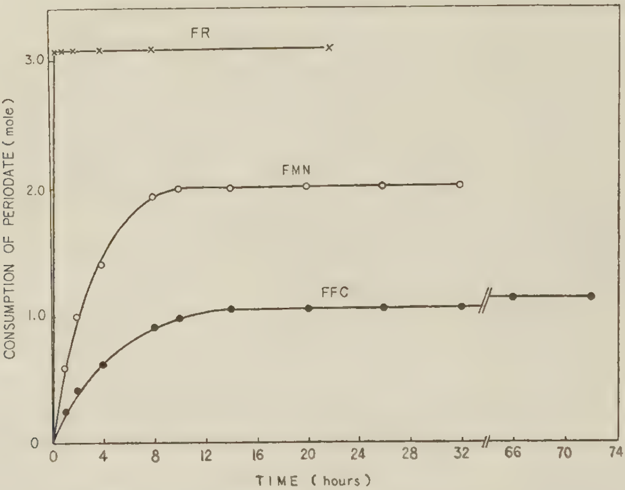

FIG. 1. Consumption of periodate by flavins. Mole of periodate consumed per mole of flavin calculated on the basis of isoalloxazine nucleus after the indicated period of reaction was shown.

This identification was further fortified by the esterification of this substance in dry methanol saturated with hydrogen chloride. The ester was extracted with chloroform and then obtained as yellow needle crystal. This compound was identified by paper chromatography with methyl ester of 6,7-dimethyl isoalloxazine-9-acetic acid (*10*). ($R_f=0.40$ by 5 per cent disodium hydrogen phosphate, and $R_f=0.63$ by *n*-butanol : acetic acid : water (4 : 1 : 5, *v*/*v*)).

From these results, the primary substance from FFC by periodate oxidation is considered to be 6,7-dimethyl isoalloxazine-9-acetaldehyde. Accordingly, it is concluded that FFC is oxidized by periodate between 2′ and 3′ carbon of its ribitol chain. Thus, chemical structure of FFC was determined to be (I), riboflavin-4′,5′-cyclic phosphoric acid.

The behavior of FFC to the enzymic reaction is interested. For example, FFC is not dephosphorylated by alkaline phosphomonoesterase (*11*), whereas FMN is easily attacked by this enzyme (*12*).

The detailed report of this communication will be presented in the future.

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LETTERS TO THE EDITORS

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ENZYMIC PHOSPHORYLATION OF 4-METHYL-5-β-HYDROXYETHYLTHIAZOLE AND THIAMINE SYNTHESIS*

Harris and Yavit (*1*) reported for the first time the enzymic synthesis of thiamine from Th** and OMP in the presence of ATP, Mg^{++} and a cell-free yeast extract. Later, Fujita *et al.* (*2*) and Leder (*3*) confirmed the above result and further suggested that both the thiazole and the pyrimidine fragments might be activated before their condensation. Fujita *et al.* (*4*) also clarified that thiamine or TMP could be formed at a greater rate from OMP-PP and ThMP without addition of ATP and Mg^{++}. In this paper, we prepared S^{35}-labeled Th and ThMP, and obtained direct evidences for the following two reactions.

$$\text{Th-S}^{35} \xrightarrow{\text{ATP, Mg}^{+}+} \text{ThMP-S}^{35}$$

$$\text{ThMP-S}^{35} + \text{OMP-PP} \rightarrow \text{TMP-S}^{35} \longrightarrow \text{Thiamine-S}^{35}$$

The enzyme used was prepared by the essentially same method as in the report of Fujita *et al.* (*2*). Th-S^{35} was incubated with ATP and Mg^{++} in the presence of the yeast enzyme, and after the reaction mixture was deproteinized with perchloric acid, the product was identified from its radioactivity and UV-absorption after separation by paper chromatographic and electrophoretic methods. Figs 1 and 2 clearly demonstrated the formation of radioactive ThMP, no other active product being detected, and the amount was proportional to the incubation time at least for the first two hours. In order to detect minor products, if formed, the reaction was carried out on a large scale and the product was subjected to ion exchange chromatographic identification. Before the chromatography, authentic samples of ThMP and ThDP were added as carriers. In this case, also, as shown in Fig. 3, two radioactive peaks appeared, but the product was only one because one peak was of unchanged Th and the other was of ThMP. The ThDP fraction showed no radioactivity. The Th and ThMP fractions (after removal of ammonium formate by passage through Dowex 50 (H^{+}) column and subsequent ether extractions) were confirmed by paper chromatography to contain only Th and ThMP as radioactive component, respectively, although the latter fraction was contaminated with an adenylic

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* This report was presented at the 111th Meeting of Japan Vitamin B Research Committee (20, Jun. 1959, Tokyo), *Vitamin*, **17**, 138 (1959).

** The following abbreviations were used. Th, 4-methyl-5-β-hydroxyethylthiazole; ThMP, its monophosphate; OMP, 2-methyl-4-amino-5-hydroxymethylpyrimidine; OMP-PP, its pyrophosphate; TMP and TDP, thiamine mono- and diphosphates.

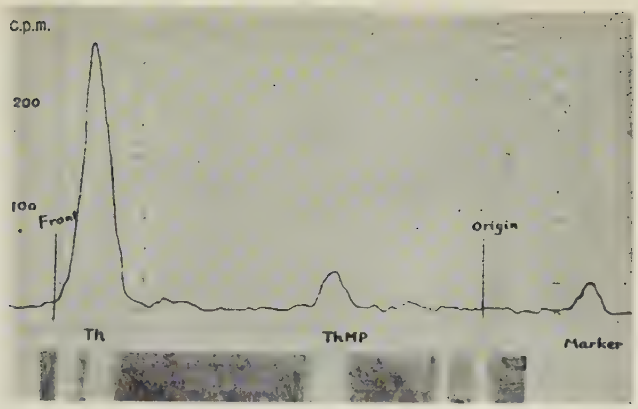

FIG. 1. Paper chromatographic identification of ThMP-S^{35}. The reaction mixture (5.1 ml.) contained 1.2 μmol Th-S^{35} (8.3×10^5 cpm.), 30 μmol ATP, 30 μmol $MgSO_4$, 30 mg. the enzyme fraction (1-3D) and 1.5 ml. McIlvain buffer, pH 7.4. Incubation time; 60 min. at 37°. Chromatography, *n*-BuOH : AcOH : water (12 : 3 : 5, *v/v*), ascending. Paper, Toyo, No. 5B. S^{35} was detected by the autoscanner, Actigraph II, Nuclear Chicago, and the photograph shows UV absorption spots (253.6 mμ).

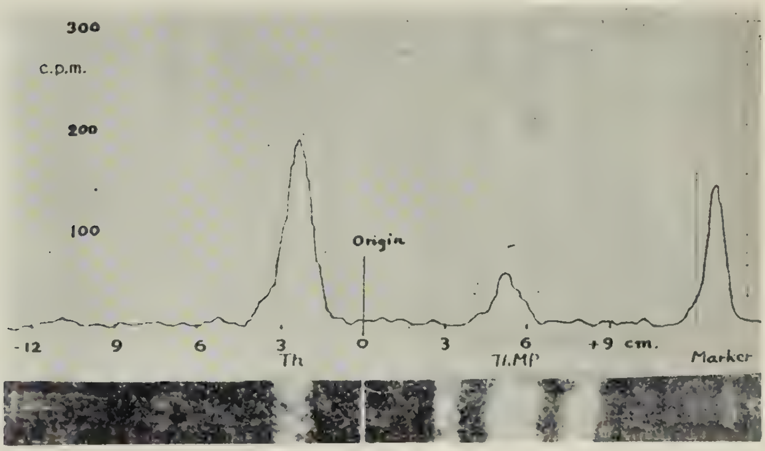

FIG. 2. Paper electrophoretic identification of ThMP-S^{35}. The sample and the detection methods are quite the same as in Fig. 1. Electrophoretic conditions; 0.1 *M* acetate buffer (pH 4.5), 14 volt/cm., 300 min. Paper; Toyo, No. 5B.

compound (presumably AMP). These results led us to the conclusion that ThMP was the only product and the phosphorylation was catalyzed by a kind of phosphokinase. Thus, our findings give a direct evidence for Fujita's assumption that ThMP might be active Th in the thiamine synthesis.

By the thiochrome method we confirmed Fujita's findings that OMP-PP* and ThMP could yield thiamine or TMP without addition of ATP.

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* OMP-PP was kindly offered by Prof Y. Nose, Kyoto Pref. Med. College, to whom we are deeply indebted.

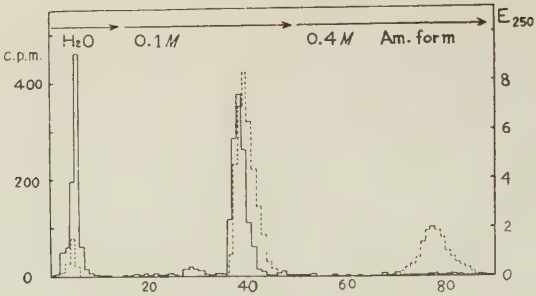

FIG. 3. Ion exchange chromatographic separation of ThMP-S^{35}. Dowex 1-×10, formate, 200-400 mesh, 1×12 cm. —Radioactivity --- E_{250}. The sample is the deproteinized reaction mixture containing 3 μmol. Th-S^{35} (2.1×10^6 cpm.), 100 μmol. ATP, 100 μmol. $MgSO_4$. 200 mg. of the enzyme fraction (1-3D) and 7.5 ml. of McIlvain buffer (pH 7.4). Total vol., 20 ml. Incubation time, 2 hrs. at 37°. 2 mg. each of ThMP and ThDP was added as carriers.

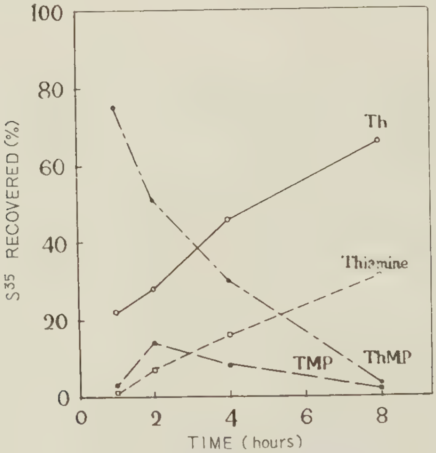

FIG. 4. Per cent recovery of S^{35}-substances during the enzyme reaction with ThMP-S^{35} and OMP-PP. The reaction mixture (5.0 ml.) contained 8.7 μmol. OMP-PP, 3.0 μmol. ThMP-S^{35} (5.3×10^5 cpm.), 50 mg. yeast enzyme (No. II-ID) and 1.5 ml. Tris maleate buffer (pH 7.6). The figures were obtained by planimetry of the autoscannings of the chromatograms (i-PrOH : i-AmOH : i-butyric acid : 28% ammonia : water (7.2 : 2.5 : 12.0 : 7.5 : 0.2 *v/v*)) and of the electropherogramms (conditions, same as in Fig. 2).

We further intended to ascertain this fact isotopically using ThMP-S^{35}. In the course of these studies we met an anomalous chromatographic run of thiamine in the *n*-butanol solvent system, but this difficulty was overcome by means of a combination of another chromatographic system (i-butyric solvent) (*5*) and paper electrophoresis. Thus, we confirmed the formation of radioactive TMP and thiamine in the two-hour incubation mixture, and TMP was no longer present after eight-hour incubation, only thiamine and Th being recovered (Fig. 4). These results permitted us to infer the reaction sequence in which TMP was first formed by the condensation and then hydrolyzed to thiamine by a phosphatase presumably present in our crude enzyme preparation.

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MECHANISM OF L-THREONINE SYNTHESIS FROM O-PHOSPHOHOMOSERINE

As reported previously, O-phospho-L-homoserine (PHS), which was formed from L-homoserine and ATP by yeast homoserine kinase, was found to be converted to L-threonine and orthophosphate (Pi) in the presence of a yeast enzyme (*1*, *2*), for which we tentatively proposed the name "PHS-phosphatase-isomerase" (*3*). Recently Wormser & Pardee called the enzyme "Homoserine phosphate mutaphosphatase" (*4*). The transfer of hydroxyl group from γ-C to β-C with concomitant dephosphorylation involved in the reaction is a subject of considerable interest. In the present paper evidences are presented that the reaction is pyridoxal phosphate (PALP)-dependent, and that the reaction proceeds with cleavage of the C-O bond in the C-O-PO_3H linkage of PHS.

The enzyme prepared from acetone-dried yeast as described in the previous paper (*1*) was further purified by adsorption on calcium phosphate gel at pH 6.2 to 6.5 and elution by ammonium oxalate followed by reprecipitation with ammonium sulfate at 0.55 to 0.65 saturation. On incubating the enzyme with PHS which was prepared as previously (*2*) and purified by chromatography on Dowex-50, threonine was formed concomitantly with the liberation of virtually equivalent amount of Pi, as determined by the periodate method (*5*) and Allen's method (*6*), respectively. Optimum pH was 7.0 to 7.5 either in phosphate or veronal buffer. The Michaelis constant, K_m, for PHS was of the order of 10^{-4}. By the addition of PALP to the reaction mixture the reaction was stimulated 1.5 to 3-fold at every step of the purification. Though the attempts to resolve the enzyme further with respect to PALP were unsuccessful, the involvement of PALP in the reaction as a coenzyme may be inferred from the following data: (a) the reaction was inhibited by the compounds which can react with PALP, such as hydroxylamine, hydrazine and KCN (Table I), as in the case of PALP-dependent amino acid decarboxylases reported by Taylor & Gale (*7*); (b) boiled extract of the purified enzyme activated "resolved" tyrosine decarboxylase from *Streptococcus faecalis* as much as authentic PALP. These data are compatible with the finding of Nisman *et al.* that the threonine synthesis from homoserine in crude extract of *E. coli* required PALP and ATP for its maximum activity (*8*).

The reaction was then followed in O^{18}-H_2O, in order to determine whether the oxygen attached to P-atom of PHS or of the water supplies the threonine-OH during the transformation of PHS into threonine. Ordinary PHS was incubated in O^{18}-H_2O with the enzyme and the necessary additions, and Pi formed was isolated at its salt, purified by reprecipitation, and

TABLE I

Effect of Various Inhibitors on Threonine Formation

Inhibitors	Concentration (m*M*)	Inhibition per cent of	
		Threonine Formation	Pi Formation
KCN	10	21	21
Azide	10	20	22
Hydrazine	1	77	66
Hydroxylamine	1	85	89
Semicarbazide	10	21	15
	1	6	4*
$FeSO_4$	10	63	72
p-Chloromercuribenzoate	1	73	58
Monoiodoacetamide	10	1*	4*
Ethylenediamine tetraacetate	10	0	5*
α, α'-Dipyridyl	10	2*	3*
NaF	10	0	0

The reaction system contained 3 μM PHS, 50 μM veronal (pH 7.3), enzyme (about 0.1 mg.-N), and 1 or 10 μM inhibitor in total volume 1 ml. Temp. 37°. *Significance is doubtful.

TABLE II

O^{18} Content in Inorganic Phosphate Released in Threonine Formation

Exptl. No.	Atom % excess O^{18} in medium water	Atom % excess O^{18} in Pi formed		
		Found	Calculated for the splitting of	
			P-O	C-O
1	1.426	0.002	0.178	0
2	1.426	0.004	0.178	0

The reaction system of exptl. No. 1 contained 1 mM PHS, 1 mM veronal (pH 7.2), 0.4 mM ethylenediamine tetraacetate, 0.2 mg. PALP, and lyophylized enzyme in 20 ml. of H_2O^{18}. Incubated at 37° for 200 minutes. Threonine and Pi formed were 0.341 and 0.346 mM, respectively. Scale of exptl. No. 2 was half of No. 1, and incubated for 240 minutes. Threonine and Pi formed were 0.272 and 0.296 mM, respectively.

subjected to analysis for O^{18} content essentially according to the method of Cohn (*9*). Should organic phosphate be cleaved at the P-O bond, one of the four O-atoms attached to the P-atom would have been replaced by O^{18} from the water. The result in Table II shows no significant incorporation of O^{18} into the Pi formed, indicating that the reaction occurred with cleavage of the C-O bond in the C-O-P linkage of PHS, and the phosphate group was intact throughout the reaction. The hydroxyl group of threonine formed was, therefore, derived presumably from the water.

Function of PALP has been elucidated by Snell *et al.* (*10*) generally in the metabolic reactions of amino acids which have electronegative atom or group at β- or γ-position. The dephosphorylation of PHS in its conversion to threonine may be explained by a mechanism similar in type to those described by them, and the threonine formation is regarded as proceeding by nucleophilic attack of water-OH on β-C of the intermediate which is formed by dephosphorylation of PHS.

Full details of the experiments will be published elsewhere.

We are grateful to Dr. S. Epstein and Dr. H. Borsook of the California Institute of Technology for giving us facilities for the analysis of O^{18}.

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TRANSFER OF RADIOACTIVE HEME AND FORMATION OF HEMOGLOBIN

The active principle to incorporate iron into protoporphyrin has been extracted from the stroma of duck erythrocytes with sodium cholate and purified by passing through Amberlite IRC 50 column to remove hemoglobin. The possibility was discussed in the preceding paper (*1*) that the radioactive heme formed by this factor combine with the acceptor to form hemoglobin which was identified chromatographically.

Schwartz and others in Wintrobe's laboratory have reported that the supernatant of chicken hemolyzate which was inactive by itself activates the iron incorporation by the preparation which was obtained by treating the erythrocyte stroma with a Waring blendor (*2*). The activation was confirmed by the authors using the resin-treated preparation. Moreover, the hemolyzate of human or horse which was also inactive by itself showed similar potentiating activity (Table I). It appears that there exists some cofactor in avian erythrocyte supernatant and in horse and human hemolyzate as well.

If this cofactor serves as an acceptor of heme and if the acceptors in different erythrocytes are different, the products to which the radioactive heme attaches may be different according to acceptors and can be separated each other. Hemoglobins of horse and duck can be easily characterized by chromatography on Amberlite IRC 50 and this advantage was used for the analysis. The cholate extract was incubated after the addition of the supernatant of duck or horse hemolyzate. The incubated samples were chromatographed and both hemoglobin content and the radioactivity of heme in each fraction were estimated. As shown in Fig. 1, most of the radioactive heme belonged to duck hemoglobin if duck supernatant was added, whereas it belonged to horse hemoglobin if duck supernatant was replaced by horse supernatant. These experiments may give some evidence that the heme synthesized by the enzyme in duck erythrocyte stroma is then transferred to globin which exists in the supernatant of the hemolyzate. The presence of free globin in the hemolyzate of duck as well as human and horse is suggested.

Although the identification of free globin were not yet successful, it was indicated that the acceptor in the supernatant was similar to hemoglobin in ammonium sulfate fractionation, but not identical with hemoglobin, for example horse hemoglobin crystals which was prepared by dialysis against distilled water was less active than its mother liquid (exp. 3., Table I) and the peak of the potentiating activity preceded that of hemoglobin by chromatography on Amberlite IRC 50 (Fig. 2).

TABLE I

The Activation of the Iron-Incorporation by the Supernatant

	Radioactivity (c.p.m.)
Exp. 1	
Cholate extract	642
Resin-treated prep.	320
,,+duck supernatant.	3940
Exp. 2	
Cholate extract	270
,,+duck supernatant.	3370
,,+human supernatant.	3480
Exp. 3	
Cholate extract	1100
,,+cryst. horse Hb.	1630
,,+mother liquid	3360

The preparation in phosphate buffer, pH 7.4, was incubated at 37° for 4 hours with $Fe^{59}Cl_3$ about 1×10^5 c.p.m, ferric ammonium citrate 2 μg as iron, protoporphyrin 0.2 μmole, cysteine 1 μmole in total volume of 5 ml. The amount of the supernatant was about 15 mg. as hemoglobin. After incubation, hemin was crystallized by adding carrier hemoglobin (*3*). The radioactivity was measured by a well-type scintillation counter and corrected for the recovery of hemin.

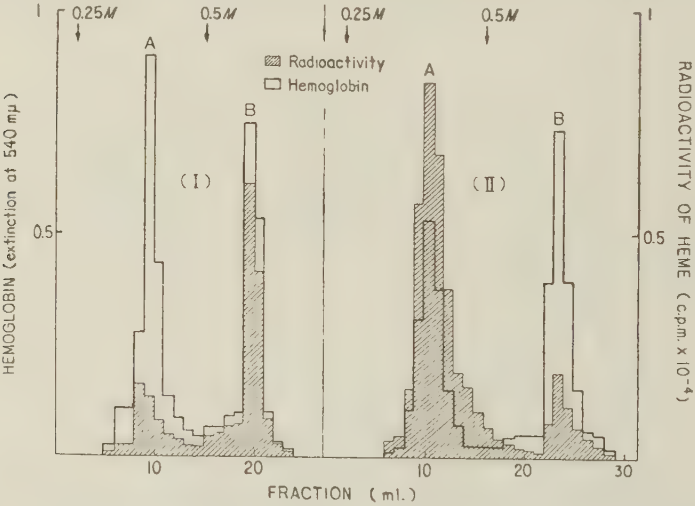

FIG. 1. The chromatogram of radioactive hemoglobin formed by the reconstructed systems. (I) Incubated with the duck supernatant. The horse supernatant was added after incubation. (II) Incuvated with the horse supernatant. The duck supernatant was added after incubation. The conditions of incubation were as in Table I, except radioactive iron of 5×10^5 cpm. was added. The column of Amberlite IRC 50, 1.3×9 cm. equilibrated with citrate buffer, pH 6.5 (salt concentration 0.2 *M*) was used (*4*). Two ml. of the sample saturated with carbon monoxide was placed on the column and hemoglobins were eluted with 0.25 *M* and 0.5 *M* buffers. Peak A corresponds to horse hemoglobin and peak B to duck hemoglobin. Heme was extracted by acetone-HCl and transferred to chloroform (*5*).

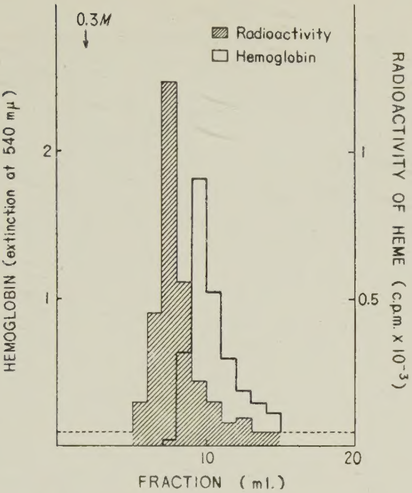

FIG. 2. Chromatogram of the factor which accelerates the iron incorporation by the cholate extract. The column of Amberlite IRC 50, 1.3×10 cm. treated as in Fig. 1 was used. Two ml. of the duck supernatant (in oxygenated form) was placed on the column and eluted with 0.3 *M* buffer. The eluates (1 ml.) were added to the cholate extract and incubated with radioactive iron (1×10^5 cpm.), protoporphyrin (0.2 μmole) and cysteine in (1 μmole) in total volume of 5 ml. (pH 7.4) for 10 hours at 37°. The estimation of the radioactive heme was as in Fig. 1. The dotted line indicates the level of the radioactivity without the addition of the factor.

Addendum to the proof:

After the manuscript has been prepared and subjected to the publication, we have received a letter and a photocopy of a galley proof to be published in *Biochimica et Biophysica Acta* from Dr. H. C. Schwartz, in which he communicated on the stimulation of heme synthesis by globin. He also observed that the supernatant of human hemolyzate stimulated the synthesis of heme.

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